Resistance mechanisms to gemcitabine in pancreatic cancer

Kulbir Mann

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by *Kulbir Singh Mann*

1st March 2021

Table of Contents

Dedication and Acknowledgements

This body of work is dedicated to every person who has suffered with pancreatic cancer, a cause not only this thesis is devoted to, but one that I dedicate my life to.

The opportunity was provided by Professor Paula Ghaneh who had the faith and confidence in me, that I could, not only successfully complete this PhD, but pursue a career in pancreatic surgery. For that I am forever grateful.

I would like to acknowledge the enthusiasm and inspiration of Professor William Greenhalf who, even before taking on this project, challenged me in ways I had not experienced. The intellectual pursuit of scientific adventure was one I never truly embarked upon and was as imaginative and exciting as anything else I have encountered.

I can only hope to maintain that passion and pass it on to younger scientists and surgeons as we try to make the lives of people with pancreatic cancer that significantly easier and longer.

I would like to thank all the PhD and post-doctoral scientists who have taken their time to train me and help me to explore my own ideas. These include Lawrence Barrera, Lucy Oldfield, Anthony Evans, Karen Aughton, Nils Elander and the MRes students who have helped diligently throughout their studies, Bethan Rogoyski and Viktoria Chatzipavli. I would also like to thank Professor Eithne Costello-Goldring, Dr Carlos Rubbi and Dr Jason Parsons who helped me to understand new techniques and allowed the time for me to be trained by their groups.

Abstract

Treatment of pancreatic cancer involves combinations of therapeutics including use of the chemotherapy agent gemcitabine. This thesis aimed to identify gemcitabine resistance mechanisms in pancreatic cancer and so enable discovery of biomarkers to tailor chemotherapy. Clonally and adaptively resistant pancreatic cancer cell lines were developed, producing five clonally resistant cell lines (Suit-2R, Suit-2R2, Suit-2R3, Suit-2R4, Panc-R), and an adapted line (Suit-2G+). Removing the drug from Suit-2G+ restored sensitivity, giving Suit-2G- cells. Gemcitabine can cause double strand DNA (dsDNA) breaks by incorporation into DNA and as a result of replicative stress induced by deoxynucleotide (dNTP) starvation. dsDNA breaks were assessed with neutral comet assays, using gemcitabine or gamma radiation. All resistant cell lines had less damage than their parent cell line did at low doses of gemcitabine; Suit-2R2 had increased dsDNA breaks at high (250nM) levels. Suit-2G cells were less sensitive to radiation and for Suit2-R2, R3 and R4 it was greater, with the greatest increase in R4. Gemcitabine starves cells of dNTPs by inhibiting ribonucleotide reductase (RR). Suit-2R and Suit-2R4 had low levels of the RR subunit RRM1, Suit-2R2, Suit-2R3, Panc-R and Suit2G+ had higher levels. Replicative stress caused by gemcitabine incorporation, due to dNTP starvation or dsDNA breaks, can be prevented by checkpoint arrest. Expression of the checkpoint protein p21 was reduced in all resistant Suit-2 lines but increased in Panc-R. Chk1 was reduced in Suit-2R and increased in Suit-2R3, R4 and Panc-R. Suggesting that in Suit-2R there was a reduction in need/ability to activate checkpoints, while in Suit-2R3 and R4 checkpoint arrest had become predominantly Chk1 dependent (more G2/M focused). Replicative stress can also be reduced by decreasing Cyclin D1 or by relative increase in the D1b isoform (slowing S-phase entry). Panc-1 is homozygous for G870A of cyclin D1 and so has more D1b, whereas Suit-2 is heterozygous. Suit-2G+/G- had increased D1a. Suit-2R and Suit-2R2 have reduced D1 with a higher proportion of D1b. Suit-2R3 and Suit-2R4 have higher D1a. Cell cycle analysis tied together these results and suggested distinct resistance mechanisms. **A:** Suit-2G+ has a normal cell cycle; increased nucleotide pools overcome replicative stress. **B:** Suit-2R, Suit-2R2 pause at G1/S, preventing S-phase entry. Starved nucleotide pools in Suit-2R, and low Cyclin D1 and Chk1 inducing an arrest in Suit-2R2. **C:** Suit-2R3/R4 have many viable cells in G2, driving through S phase accepting replicative stress. **D:** Panc-R has a measured and controlled cell cycle through high RRM1.

The different ways of achieving resistance identified in cell lines may have equivalents in patients, identification of the dominant mechanism can be exploited as a therapeutic biomarker.

List of Abbreviations

5-FU	5-fluorouracil
ATM	Ataxia-telangiectasia-mutated protein
ATR	Ataxia telangiectasia Rad3-related proteins
BCA	Bicinchonic Acid
BR	Borderline resectability
Ca 19-9	Carbohydrate antigen 19-9
CDA	Cytidine deaminase
CDC	Complement dependent cytotoxicity
CDK	Cyclin dependent kinases
dCK	Deoxycytidine kinase
DDR	DNA damage response
dFdC	2',2'-difluoro 2' deoxycytidine
dFdCDP	2',2'-difluoro 2'-deoxycytidine diphosphate
dFdCTP	2',2'-difluoro 2'-deoxycytidine triphosphate
DMSO	Dimethyl sulfoxide
dNTP	Deoxyribonucleotide triphosphate
dNDP	Deoxyribonucleotide diphosphate
dsDNA	Double strand DNA
DSB	Double strand breaks
DTCD	Deoxycytidylate transaminase
E2F	E2 transcription factor
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
ERCC1	Excision repair cross-complementation 1
ESPAC	European Study Group for Pancreatic Cancer
FACS	Fluorescence activating cell sorting
FBS	Foetal bovine serum
FDG	^{18}F-fluoro-2-deoxy-D-glucose
FOLFIRINOX	5-fluorouracil, leucovorin, irinotecan and oxaliplatin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEMCAP	Gemcitabine and Capecitabine
hENT1	Human equilibrative nucleoside transporter 1
MD-CT	Multi-detector computed tomography
MDT	Multi-disciplinary team
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
NSCLC	Non-small cell lung cancer
NICE	National Institute of Clinical Excellence
NF-kB	Nuclear factor kappa-light-chain-enhancer of activated B cells
nM	Nanomolar
NRF-2	Nuclear factor erythroid 2-related factor 2
PAK	p21 associated kinases
PET	Positron emission tomography
PI	Propidium Iodide
RB	Retinoblastoma protein
RPMI	Roswell Park Memorial Institute
RR	Ribonucleotide reductase
RRM1	Ribonucleotide reductase subunit 1
RRM2	Ribonucleotide reductase subunit 2

RT	Reverse transcriptase	SDS	Sodium dodecyl sulphate
RT-PCR	Quantitative real-time polymerase chain reaction	TNM	Tumour, Node, Metastasis
		Top1	Topoisomerase 1

Aim

To identify innate mechanisms of resistance to gemcitabine through exposure in pancreatic cancer cell lines and contrast with acquired mechanisms of adaptation during gemcitabine treatments

Objectives

a) To generate gemcitabine resistant pancreatic cancer cell lines
b) To assess the growth rates of control and resistant pancreatic cancer cell lines
c) To compare the expression of gemcitabine molecular targets in sensitive control and resistant pancreatic cancer cell lines
d) Induce transient resistance to gemcitabine by exposure to gemcitabine and verify loss of resistance through removal of drug
e) Assess the cytotoxic effects of gemcitabine by analysis of double stranded DNA damage in all cell lines
f) Assess the cytostatic effects of gemcitabine through mRNA transcript and protein levels analysis of the ribonucleotide reductase complex sub-units in all cell lines
g) Explore the effect of knockdown and transfection of RRM1 transcript on growth and resistance to gemcitabine in all cell lines
h) Assess the effect of gemcitabine resistance on the cell cycle checkpoint control markers p21, CyclinD1 and Chk1 in all cell lines
i) Analyse the combined effects of gemcitabine on the cell cycle phases of all control and resistant cell lines, relating them to DNA damage, nucleotide resources and cell cycle regulation

1. Introduction

Pancreatic cancer is one of the few malignant diseases that has progressed little with respect to overall survival. Even though surgery is the mainstay of curative treatment, it is offered to relatively few patients (1, 2). Therefore, the role of chemotherapy is of significant importance, not necessarily through a curative strategy but in order to prolong life. Gemcitabine has formed a critical part of adjuvant and palliative chemotherapy for many years, and remains so through multiple randomised clinical trials reviewing combinations and alternative agents (3-6). With a disease that affords few curative surgeries, resistance to chemotherapy agents brings exceptionally poor survival. It is important that patients have at least one option of effective treatment, and that premise forms the rationale of this thesis.

1.1 Incidence and prevalence of pancreatic cancer

The awareness of pancreatic cancer is slowly increasing worldwide, with the number of annual deaths from the disease expected to exceed that of breast cancer in Europe, over the next few years (7). Across the world the incidence of pancreatic cancer was 458,918 in 2018 with 432,242 mortalities, giving an age standardised incidence rate of 5.5 per 100,000 and a mortality rate of 4.8 per 100,000 (8). It predominately occurs across Asia and Europe with incidence proportions of 46.7% and 28.9% respectively. There is a slight male preponderance with incidence rates per 100,000 of 5.5 in men and 4.0 in women (8). The five-year survival rates range between 3% and 9% across the world (2, 9, 10).

1.2 Pathology and Staging

The majority of pancreas carcinoma is ductal adenocarcinoma which accounts for over 85% of tumours. For lesions that obstruct the distal bile duct, clinical presentation and investigations are the same and often lead to identical surgical management. The differential includes distal cholangiocarcinoma, duodenal carcinoma, and peri-ampullary malignancy, which may be intestinal or pancreatic type. The size of the tumour and extent of local invasion and distant metastatic spread are of paramount importance in determining management strategies, especially with the advent of neoadjuvant clinical trials. The staging process of pancreatic cancer is through the TNM classification of malignant tumours maintained by the Union of International Cancer Control (11).

1.3 Clinical diagnosis

The retroperitoneal location of the pancreas reduces the effect a mass has on the gastrointestinal tract and lesions become quite sizeable before they cause any symptoms. If they occur at the head of the pancreas, the first noticeable symptom may be jaundice as the distal bile duct becomes obstructed. This partly explains why only 10% of patients are able to undergo curative surgery as other insidious vague symptoms delay referral and diagnosis (12). Patients complain of non-specific symptoms of weight loss, change in appetite, early satiety and vague back pain. Specific complications of the disease include obstructive jaundice, malabsorption and late onset diabetes (1). The relative lack of specific symptoms and physical signs delay prompt investigation.

1.4 Investigations and pathological diagnosis

There are no serum markers specific to pancreatic cancer but carbohydrate antigen nineteen nine (Ca 19-9) is used for many pancreas related conditions. The use of Ca 19-9 as a diagnostic marker has become standard across the world with a specificity and sensitivity of 80% and 75-80% respectively (13, 14). It is a cell surface antigen glycoprotein expressed on pancreatic cancer cells but is also raised in other conditions such as acute and chronic pancreatitis, obstructive jaundice, cholangitis, liver cirrhosis and bile duct malignancy (15). There is some evidence it can be used as a prognostic marker and it is routinely used for post-operative follow-up (1, 16-18). Imaging is often in the form of cross-sectional imaging and multi-detector computed tomography (MD-CT) has a sensitivity of 76-100%, a specificity of 72% and a positive predictive value of 89%, it is recommended by many guidelines for diagnosis and staging (19-22). Positron emission tomography (PET) utilises the radiotracer ^{18}F-fluoro-2-deoxy-D-glucose (FDG) which builds up in cells with high metabolism, particularly if (as with cancer cells) they are not metabolising glucose through the Krebs cycle, and is used for detecting extra-pancreatic disease. Its role in refining staging has led to it being used in conjunction with MD-CT as part of national clinical guidance (19, 20, 23). Endoscopic ultrasound and fine needle aspiration are being more utilised to produce high resolution images, especially with lesions smaller than 3cm (19, 20, 24). It has the ability to obtain cells for cytology and even fragments of tissue for histology and may have a role in detecting biomarkers for tailoring chemotherapy regimens.

1.5 Surgical management

Though there are promising studies trialling neoadjuvant chemotherapy for pancreatic cancer, surgical resection is the mainstay of curative management. Even so only 10% of patients had surgery in 2013-2014 within the United Kingdom and the median survival of patients is 11-23 months and 10-

27% are alive at five years (1, 25). The key decision regarding surgery lies with the precise location of the tumour and its borders with surrounding vascular structures. The definitions of borderline resectability (BR) have been established by an international consensus in 2016, in order to standardise reporting and MDT decision making (26). They defined BR with respect to anatomical, biological and conditional factors and extended features of the disease to incorporate additional factors that make surgery high risk. The concerning radiological features are that of stenosis, deformity, narrowing or occlusion of major vessels and concerning biological features include presence of metastatic disease and lymph node disease. The final conditional features are those regarding the patient and their fitness and suitability for operative intervention (26). Whether the patient has surgery or not, there is certainly a role for palliative and adjuvant chemotherapy and, with the completion of recent trials, neoadjuvant too.

1.6 Adjuvant and Neoadjuvant therapy

Over the past twenty years multicentre randomised control trials have been the mainstay of pancreatic cancer research and evolving management regimes. The European Study Group for Pancreatic Cancer (ESPAC) have produced multiple randomised trials beginning with ESPAC-1 published in 2004 that found adjuvant chemotherapy led to a five-year survival of 21% compared to 10% in the chemoradiotherapy group and 8% in the observation group (6). The ESPAC-3 trial demonstrated adjuvant gemcitabine was similar in survival to 5-fluorouracil/folinic acid but had a lower toxicity profile, becoming the standard of care for a long period of time (4). ESPAC-4 randomised patients into two treatment paradigms: gemcitabine vs gemcitabine and capecitabine (GEMCAP) and found a median survival advantage of 28 months over 25.5 months with the combination group (5). This study was particularly important as it started a trend of combination chemotherapy for pancreatic cancer. A Canadian and French group performed a multicentre randomised control trial reviewing gemcitabine compared with a modified dose combination of 5-fluorouracil, leucovorin, irinotecan and oxaliplatin (FOLFIRINOX) (27). They discovered a median survival of 54.4 months in the FOLFIRINOX group compared to 35 months with gemcitabine. There was a greater proportion of serious adverse events, 75.9% with FOLFIRINOX compared to 52.9% with gemcitabine (27). At present the NICE (National Institute of Clinical Excellence) guidelines do not recommend FOLFIRINOX as adjuvant treatment but GEMCAP, and for those not well enough to tolerate combined six cycles of treatment, gemcitabine monotherapy (21).

Gemcitabine has not been incorporated as a single agent for neoadjuvant treatment in any randomised controlled trials, but has been retrospectively analysed (28-30). Many recent trials have

found utility with FOLFIRINOX in borderline and locally advanced pancreatic cancer patients and a few meta-analyses have demonstrated this (31-33). The general outcomes are that there are up to 60% increases in resectability in tumours that were high risk and there is a marked improvement in downstaging and complete margin free resections. This has led to the conclusions that neoadjuvant treatment with FOLFIRINOX does lead to increased survival and improved histological outcomes in patients with locally advanced or unresectable pancreatic cancer (31-33). There has yet to be a phase III trial but ESPAC-5F was a phase II randomised multicentre trial with four arms: primary surgery, neoadjuvant FOLFIRINOX, neoadjuvant GEMCAP and neoadjuvant chemoradiotherapy. Of 88 patients, all neoadjuvant treatments resulted in increased one year survival with 77% alive at one year compared to 40% in the straight to surgery group (3). Though we are waiting long term outcomes, it would appear that the future of neoadjuvant chemotherapy is almost here. The combinatorial drug strategy has been pursued because of the poor outcomes with adjuvant monotherapy and the degree of resistance that exists.

Though we can accurately stage and diagnose patients, tumour heterogeneity prevents predictability of chemotherapeutic treatment. We are unable to delineate which patient that receives GEMCAP or gemcitabine will respond giving a survival advantage, and this applies to the neoadjuvant FOLFIRINOX trials in predicting tumour downstaging. Though a multi-drug regimen is unlikely to have a unified resistance to all the agents, there will be a degree of cross-resistance mechanisms. Even that is beyond the current evidence base as biomarkers to predict gemcitabine resistance have not yet been established.

1.7 Palliative chemotherapy

In the setting of metastatic pancreatic cancer, gemcitabine has historically played a major role and now has been trialled with and against more promising regimens such as FOLFIRINOX and nab-paclitaxel. The PRODIGE4/ACCORD 11 enrolled 342 patients and found median overall survival was significantly improved with FOLFIRINOX, 11.1 months vs 6.8 months with gemcitabine but the former group had poorer outcomes if they were confined to bed during a significant part of the day (34). Long term follow-up of the phase III MPACT trial of 861 patients found a significant increase in survival when gemcitabine was combined with nab-paclitaxel compared to gemcitabine alone, 8.7 vs 6.6 months (35). NICE guidance recommends FOLFIRINOX in patients that have a good performance status and gemcitabine in combination with nab-paclitaxel for this who do not. If patients suffer adverse effects from combinatorial therapy then gemcitabine alone is then advocated (21).

1.8 Principles of chemotherapy resistance

The general principles of resistance are thought to be either primary or secondary, where patients either fail to respond to therapy completely or gradually the response diminishes over time. The primary resistance mechanisms are thought to be associated with the tumour microenvironment, cell signalling or aberrant apoptotic pathways. Secondary resistance involves clonal selection, initiation of cell cycle arrest, downstream marker activation or senescence and genetic mutation (36). In either group it is imperative to identify the potential biomarkers that either predict primary or secondary resistance. Then it would be possible to not only select which treatment modality is best but also the organisation of treatment. Though specifically not predictive biomarkers, tailored treatment has been achieved in breast cancer with the use of trastuzumab in HER2 positive disease and in malignant melanoma with the use of BRAF inhibitors in mutant BRAF patients (37). Developing on from this, it is then possible to consider targeted therapies that may overcome the acquired resistance and provide a second hit to the tumour. Malignant melanoma is an example where the use of MEK inhibition may overcome BRAF inhibitor resistance, but even this strategy has not translated to other cancers. Even the timing and dosing of a chemotherapeutic drug may affect its resistance and some cancers that are resistance then become sensitive in the absence of the drug. The principles of intermittent dosing and continuous dose of medications may have a role in overcoming this. Having a second agent that can induce sensitivity to a cell line, such as inducing senescence prior to using a senolytic drug is an effective tailored approach (37). This would allow multi-targeted specific chemotherapy regimens as opposed to an effective but heavily toxic combination of medications. That would be the optimal aim and given that there are only two NICE recommended treatment regimens for pancreatic cancer, and that gemcitabine is included in both of those strategies, a study of its resistance mechanisms is certainly warranted.

1.9 The metabolism and mechanism of action of gemcitabine

Gemcitabine (2',2'-difluoro 2'-deoxycytidine, dFdC) is a deoxycytidine analogue and has a dual mechanism of functions, cytostatic and cytotoxic. Practically it is given as an intravenous infusion and its uptake into the cell is an active or facilitated process, through the function of nucleoside transporters, including human equilibrative nucleoside transporter 1 (hENT1). This provides bidirectional passage and the roles of deoxycytidine kinase (dCK) and cytidine deaminase (CDA) are essential for the activation and deactivation of dFdC. There is a triple phosphorylation process initiated by dCK to produce dFdC monophosphate, which is then converted to di- and triphosphate through the actions of further pyrimidine kinases. The deamination process to inactivate the

phosphorylated and non-phosphorylated dFdC is carried out by deoxycytidylate transaminase (DCTD) and CDA respectively. This eventually forms difluorodeoxyuridine and CDA facilitates transportation of the degraded gemcitabine out of the cell.

The diphosphorylated form (dFdCDP) is responsible for the cytostatic effects of gemcitabine as it binds to ribonucleotide reductase subunit 1 (RRM1) which forms part of the ribonucleotide reductase complex (RR) to supply deoxyribonucleotide triphosphates (dNTP) for DNA replication. The binding of RRM1 prevents it combining as a tetramer with RRM2, and halts the addition of a phosphate to deoxyribonucleotide diphosphate (dNDP). There is an additional component to the RR complex, p53R2 which is expressed through DNA damage and oxidative stress through a p53 initiated pathway. p53R2 is able to substitute RRM2 in order to maintain or increase the function of RR. The triphosphorylated form (dFdCTP) forms the cytotoxic response of gemcitabine and causes direct DNA damage. As it competes with dNTPs for incorporation with DNA, there is DNA polymerase dislodgement leading to “masked chain termination”. There is also aberrant topoisomerase-I cleavage which in combination with direct incorporation induced double strand breaks. The dual action of gemcitabine causes replicative stress and induces cell cycle arrest in G1 or S phase, leading to abnormal replication or the initiation of pro-apoptotic pathways. The effects of gemcitabine are shown in Figure 1.

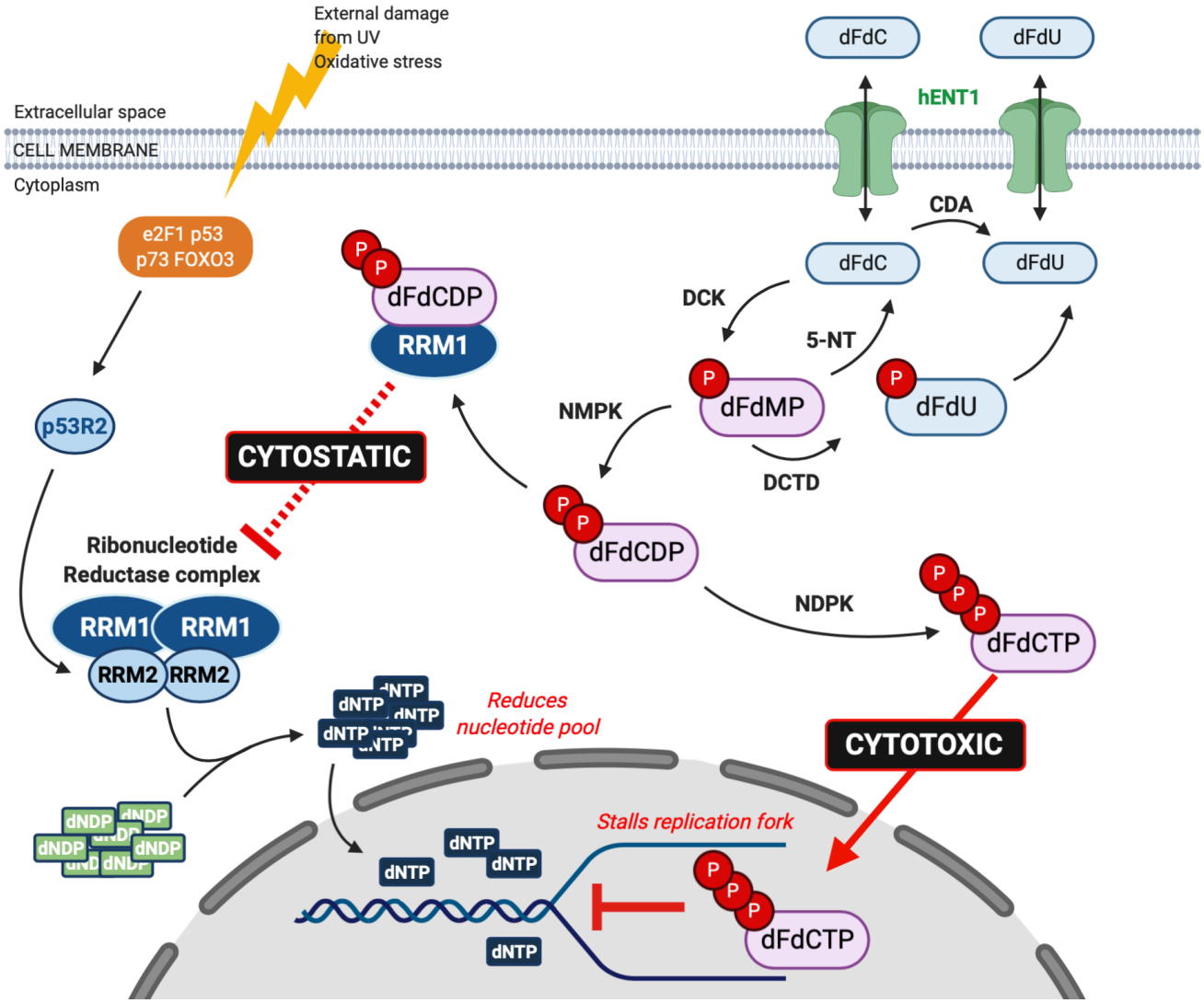


Figure 1 *Diagram depicting the phosphorylation of gemcitabine into its cytostatic diphosphorylated form (dFdCDP) and its cytotoxic triphosphorylated form (dFdCTP). (hENT1: Human equilibrative nucleotide transporter 1, dFdU: Uracil inactive metabolite, -MD, -DP, -TP: mono-, di-, triphosphate CDA: Cytidine deaminase, dCK: deoxycitidine kinase, 5-NT: 5' Nucleotidase, DCTD: Deoxycytidylate deaminase, dCTP: deoxynucleoside triphosphates, NMPK/NDPK: nucleoside mono-/diphosphate kinase, dNDP: nucleoside triphosphate, dNTP: nucleoside triphosphate)*

1.9.1 Nucleoside transporters

Gemcitabine is transported into the cell by hENT1 (amongst other transporters), in an inactive form. It stands to reason that alterations in the expression of hENT1 will affect a change in the effectivity of dFdC. There is some cell line evidence of hENT1 knockdown reducing gemcitabine uptake, by way of experimenting on low hENT1 expression through cells with natively reduced levels, through combination chemotherapeutic agents and knockdown studies. It was found that low hENT1 leads to a reduction in the effectivity of gemcitabine (36, 38, 39). Immunohistochemistry experiments on tumour microarrays and publications from our group have demonstrated that low hENT1 protein levels is predictive of a poor response to gemcitabine. There was a difference of 9.1 months in overall

survival in patients that received adjuvant gemcitabine with a non-significant advantage for low hENT1 in patients treated with 5-FU (40, 41). A meta-analysis of seven pancreatic cancer studies found hENT1 expression was significantly associated with prolonged disease-free survival and overall survival in patients receiving gemcitabine (42).

1.9.2. Activation of gemcitabine

The phosphorylation of dFdC is an essential step towards the active form and there have been many studies investigating the role of dCK in the resistance of gemcitabine. From an in vitro perspective there have been multiple developed gemcitabine resistant cancer cell lines that have shown missense and deletion mutations in dCK and reduction in its protein levels (43, 44). There has also been a described effect of NRF-2 driven antioxidant transcription from decreased dCK, that contributes to gemcitabine resistance (45). From a patient perspective there have been some associations with overall survival. RT-PCR was performed in pancreatic cancer patients receiving adjuvant gemcitabine and stratified into low and high dCK, and a 9.2 month overall survival difference was noted in patients with high dCK mRNA transcript (46). This was not seen in experiments on tumour tissue. Immunohistochemistry experiments on intestinal type cancers demonstrated a better prognosis with high dCK protein levels, but not pancreatobiliary (47). Though there is a clear hypothetical advantage to increased dCK expression, increasing sensitivity to gemcitabine, this has not been demonstrated at a clinical level.

1.9.3 Direct DNA incorporation

As gemcitabine attempts to insert its triphosphate into DNA, polymerases may pause at the 3'-terminal or 3'-penultimate position after where dFdC is incorporated (36). Exonucleases can excise mismatched deoxyribonucleotides but experiments focused on the excision of the gemcitabine nucleotide found they are difficult to excise. DNA polymerases can actually extend the 3’-dfdCMP resulting in internal incorporation of the cell (48). The excision repair cross-complementation 1 (ERCC1) gene has been theorised to be important in excising gemcitabine, with higher expression leading to resistance in non-small cell lung cancer (49). These studies progressed to clinical tissue with mRNA levels assessed in tumour tissue demonstrating that ERCC1 may predict poor survival in patients treated with gemcitabine but ultimately a phase II trial in NSCLC was halted early due to a futility analysis (39, 50). Topoisomerase I (Top1) functions by cleaving DNA and separating strands for all functions, transcription, translation, condensation and recombination. It has been discovered that reversion of Top1 cleavage complexes is slowed, leading to an accumulation of double strand

breaks (51). The only cancer that has shown reduced Top1 is acute myeloid leukaemia where resistance to gemcitabine was demonstrated. Gemcitabine may also induce reactive oxygen species through the activation of NF-kB which complements its other cytotoxic mechanisms. The nuclear translocation of Nrf-2 initiates transcription of cytoprotective anti-oxidants, and siRNA knockdown studies have demonstrated increased sensitivity to gemcitabine (52, 53).

1.9.4 Inhibiting ribonucleotide reductase

Gemcitabine not only incorporates itself into DNA, causing strand breaks but also competitively inhibits by combining with the RRM1 subunit, reducing the nucleotide pools. Many studies have reviewed the effect of the RR subunits and resistance to gemcitabine. Inhibition of RRM1 through binding of dFdCDP reduces the pool of dNTPs. At first this will increase the relative concentration of dFdCTP against dFdCTP, therefore increasing the incorporation of dFdCTP into DNA. There will be an increased cytotoxic effect and increasing the levels of RRM1 will restore the levels of dNTPs, reducing the relative incorporation of dFdCTP. Reduction in dNTPs will eventually lead to an inability to replicate DNA and thus replicative stress. Increasing RRM1 reduces this effect, however dNTP levels restoration continues DNA replication, but also allows incorporation of dFdCTP leading to DNA breaks in dividing cells (43, 54-58). RRM1 studies in the form of clinical research trials is discussed later in the thesis.

Our group have investigated which transcripts associated with gemcitabine resistance using Illumina Human RNA v3Bead and 810 Protein Antibody microarrays and developed resistant Suit-2 cell lines. It was discovered that the RRM2 subunit was differentially expressed in the resistant cell line and that its reduction at a transcript and protein level garnered resistance. Other studies looking at RRM2 have also found that increases in RRM1 and RRM2 in developed gemcitabine resistant Panc-1 cell lines reduced sensitivity (59). p53R2 is inducible by DNA damage through a p53 mediated pathway and has never been shown to be significant in pancreatic cancer. There has been a suggest that it can be used as a prognostic marker in nasopharyngeal cancer and increased mRNA expression may afford resistance to gemcitabine in cholangiocarcinoma patients (60, 61). Given its role as a substitute to RRM2 and its regulation by p53, it is reasonable to assume that p53 mutations will have an impact on gemcitabine resistance via regulation of ribonucleotide reductase. This is complicated by the fact that different p53 mutations will have varying impact by regulation of p53R2 and some mutations will reduce expression of apoptosis related genes but not p53R2. Alternate mutations will inhibit p53R2 regulation but have no impact on other cell cycle regulators and apoptotic proteins (62, 63).

1.10 Cell cycle regulation

There are many signalling pathways and checkpoint regulators that ensure propagation of successful DNA replication and arrest in order to repair or enter pro-apoptotic pathways. Gemcitabine induces stress replication and in order to overcome this, there must be either repair pathways initiated or apoptosis induced, with either strategy there will be alterations to the cell cycle checkpoints. The following markers have been found to have an important role in pancreatic tumourigenesis or have been previously investigated by our unit. They are summarised in figure 2.

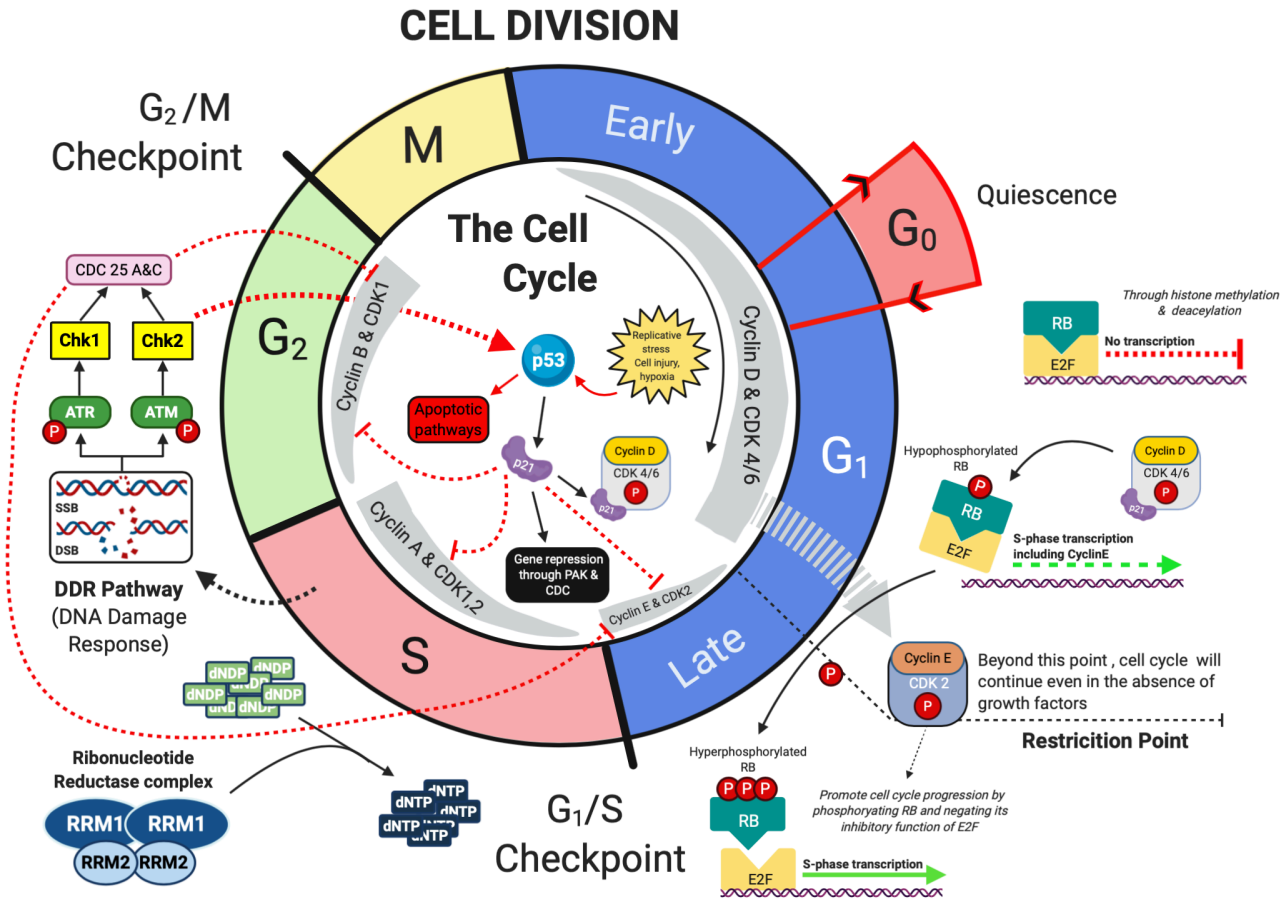


Figure 2 *A diagram depicting the cell cycle and the checkpoints with pertinent markers discussed within this thesis. (CDK: Cyclin dependent kinases, PAK: p21 associated kinases, CDC: complement dependent cytotoxicity, RB: Retinoblastoma protein, E2F: E2 transcription factor, dNDP: nucleoside triphosphate, dNTP: nucleoside triphosphate, ATM/ATR: Ataxia-telangiectasia-mutated and ataxia telangiectasia Rad3-related (ATR) proteins)*

1.10.1 p21

p21 is induced by p53 and enacts a number of effector mechanisms through the inhibition of cyclin dependent kinases and proliferating cell nuclear antigen, all required for S-phase progression. Thereby this enables cell cycle arrest, senescence, DNA repair and potentially apoptosis. Not only

does it have a direct inhibition of the cell cycle kinases it also mediates p53-dependent gene repression of cell cycle regulators including Chk1, CyclinB1, CDC2 and CDC25. p21 has a complex relationship with apoptosis and can actually act as an inhibitor in order to maintain arrest. It is theorised that post translational modifications such as phosphorylation and caspase cleavage can differentiate apoptotic and arresting pathways (64). There are p21-activated kinases (PAK) that have been implicated in oncogenic signalling pathways that involved in pancreatic cancer. PAK4 has been shown to be increased at an mRNA and protein level in multiple pancreatic cancer cell lines and a degree of co-amplification with PAK1 and CyclinD1 (65). Further experiments using gemcitabine resistant cell lines were found to express higher protein levels of PAK4 and less hENT1. If PAK4 was knocked down then sensitivity was restored (66). It has been theorised that PAK1 mediates gemcitabine resistance by suppressing DNA damage through the NF-kB pathway. It was also discovered that PAK1 was elevated and RRM1 increased in patients with pancreatic cancer (67, 68). There are numerous interactions with p21 and the functions of gemcitabine and its role in oncogenesis and chemoresistance requires further investigation.

1.10.2 Cyclin D1

The Cyclin D family regulates cyclin dependent kinases (CDK4 and CDK6 predominately) that are essential for progression through the G1/S checkpoint. In G1 it rapidly accumulates in the nucleus and then degrades once the cell enters S phase (69). Its role in pancreatic oncogenesis has been reported in many studies and that increased expression of Cyclin D1 leads to DNA replication and cell proliferation in Panc-1 and MiaPaCa pancreatic cell lines. The role of Cyclin D1 and miRNA-720 was also established in that the micro-RNA suppresses tumour growth by downregulating Cyclin D1 (70, 71). This was confirmed by the transfection of Cyclin D1 plasmids in Panc-1 cells where the effect of miRNA-720 was abrogated. The DNA encoding Cyclin D1 was found to have a polymorphism at position 870, codon 242, exon 4, where a splice variant occurs (Figure 3).

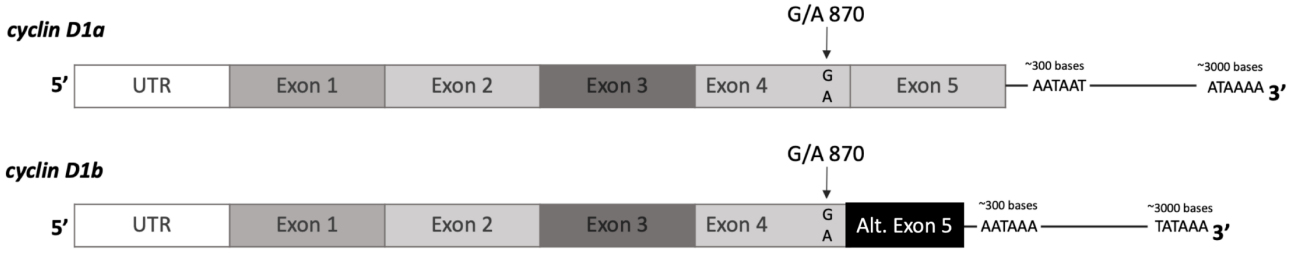


Figure 3 *Cyclin D1a codes exons 1-5 and a 3' untranslated region. Cyclin D1b encodes exons 1-4 and intron 4 with a 3' untranslated region. The expression levels of cyclin D1a/b are influenced by the existence of a G/A polymorphism at nucleotide 870, the final nucleotide of exon 4* (72)

A and G alleles provide both transcripts but the adenosine produces the aberrant transcript more frequently. The G870A polymorphism has been theorised to be associated with a risk of breast, colorectal and pancreatic cancer (72-74). The two variants produced are Cyclin D1a and D1b with the latter expressing the lighter protein. There is clearly an oncogenic role for Cyclin D1 in pancreatic cancer but its effect on gemcitabine resistance and biomarker potential has yet to be established.

1.10.3 Checkpoint Kinase 1 (Chk1)

Chk1 is another cell cycle regulator the facilitates cell cycle arrest in order to facilitate DNA repair. It functions at the G2/M checkpoint but is likely to be utilised throughout the cell cycle. It forms part of the DNA damage response (DDR), activated by Ataxia-telangiectasia-mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) proteins, with the former mainly in response to double stranded damage (75). Increased expression of Chk1 and Chk2 induce cell cycle arrest and DNA repair and clearly play a prominent role in the defence against cancer development. Chk1 has been the focus of many studies, especially in pancreatic cancer and has been linked to RRM1 and gemcitabine effectiveness. Chk1 inhibition has been a focus of clinical drug development but their role as a chemosensitizer has yet to be established. Treatment of five different pancreatic cancer lines with a Chk1 inhibitor prior to gemcitabine treatment, was shown to enhance DNA damage and cause a downregulation in the RR apparatus. This synergistic effect caused growth inhibition and apoptosis (76). Further work in pancreatic cancer cells demonstrated that there was not only a G2-M duration extension but the replicative stress was a key finding, with a high accumulation of S-phase cells with persistent genomic damage (77). Further work has been performed on drug combinations in vitro to affect the DDR pathway. The role of Chk1 is essential to DNA repair and cell cycle regulation and it has a role in gemcitabine resistance (77, 78).

Gemcitabine resistance is multifactorial and likely to have diverse strategies. The aim of this thesis is to develop gemcitabine resistant pancreatic cancer cell lines and then investigate those mechanisms. In emulating resistance from a true-to-life patient perspective, where pulsed treatments of gemcitabine are administered, there will be translatable revelations. This holds true for solid tumour masses treated with neoadjuvant/palliative gemcitabine (potentially acquired resistance) and for resected specimens and circulating tumour cells treated with adjuvant gemcitabine (potentially clonal cells demonstrating resistance). For both paradigms, investigations into the cytotoxic effects, cytostatic effects and cell cycle regulatory mechanisms will provide holistic overviews to delineating resistance mechanisms to gemcitabine.

2. Materials and Methods

General lab equipment

Heraeus Biofuge Primo Centrifuge	(Thermo Fisher Scientific)
Heraeus Pico Centrifuge	(Thermo Fisher Scientific)
Vortex Genie 2	(Scientific Industries Inc.)
TC10™ Automated Cell Counter	(Bio-Rad Laboratories)

2.1 Cell acquisition and passaging

Suit-2 and Panc-1 cell lines were obtained from stocks provided by the Greenhalf group at Liverpool University. Suit-2 is an immortalised pancreatic cancer cell line derived from metastatic liver lesions and Panc-1 has been immortalised from a native pancreatic adenocarcinoma**.** Both these cells lines have p53 and KRAS missense mutations of p.R273H and p.G12D respectively. Cells were maintained in sterile T75 vented flasks using 25ml of Roswell Park Memorial Institute (RPMI) 1640 medium enriched with sodium bicarbonate and 50ml foetal bovine serum (FBS) (10%) and 5ml 200mM L-glutamine (1%) added to every 500ml stock. These flasks were incubated at 37^{o}C, 5% CO_2 in a well-maintained air humidified incubator. Cells were inspected regularly for any evidence of infection and confluence assessed. Once they reached 80-90% confluence then a passage sub-culture was undertaken. This performed in a secondary cell culture room which contained the incubator using laminar flow biological safety cabinets under strict aseptic conditions. All reagent bottled used for culturing were allowed to reach room temperature, whereas they are normally stored at 4^{o}C. The UV light was always switched on for fifteen minutes prior to opening the front panel of the hood. The use of Trigene disinfectant and 70% ethanol was implemented to clean the base and to all apparatus and flasks brought into the hood. New sterile T75 flasks and pipettes and a clean pipette controller were used for each passage process. Each flask had all conditioned media removed and a rigorous cleaning process using 10ml of phosphate buffered saline and then a five-minute treatment of 3ml of trypsin dissociation reagent. With a firm tap cells were completely detached and a further 7ml of conditioned RPMI was administered to neutralise the trypsin. A further washing process allowed an almost complete cell population in a 10ml solution. Depending on the circumstances either a 1:10 or a 1:20 split was performed placing either 0.5ml or 1ml of cell suspension into 19.5 or 19ml of media in a fresh T75 flask.

2.2 Cell counting and cryopreservation

General Reagents

Freezing media (X2) Volume of 100ml: 10ml DMSO, 20ml FBS, 70ml RPMI-1640

Once cells were at 80-90% they underwent multiple experimental processes including cryopreservation. After the trypsination process with 7ml of RPMI additive stock added to the falcon tube, cells were centrifuged at 1000xg for 1 minute. This developed a cell pellet once the supernatant was discarded that was washed using 5ml of PBS. This was then re-centrifuged and then once supernatant discarded, a further wash with PBS performed. 10μL of this cell solution was taken and placed onto a cell counting slide for use with the TC20™ automated cell counter. The reading given was of cell numbers per millilitre of solution and guided the quantity of cells for each experiment and those cryopreserved. The PBS cell solution was then centrifuged once more and then the cell pellet washed using a freezing media which contained 500μL of stock RPMI with 500μL of freezing media (10%). The cell concentration of the media was 1 million cells/ml and divided into 1.5ml cryogenic vials and then underwent a gradual freezing process at initially -80°C and then into a polypropylene freeze box to be stored in liquid nitrogen. For resurrection a vial was taken from liquid nitrogen and allowed to thaw at room temperature with 500μL of room temperature stock RPMI media. Once liquefied the solution was transferred to a 15ml falcon tube and centrifuged at the settings above. A two stage 5ml PBS wash was performed to remove the DMSO and allow the cells to be placed into stock RPMI media in a T25 or T75 flasks and placed into an incubator. The cells were observed regularly to ensure adequate growth prior to appropriate sub-culturing. The passage number of cells was always recorded on the flask to ensure experiments were performed with low passage number cells to prevent genetic variations and drift.

2.3 Generation of gemcitabine resistant cell lines

General Reagents

Gemcitabine (SelleckChem Llc)

There were two separate methods used for generating resistant cells and the gemcitabine that was utilised was purchased from Selleck Chemicals LLC. The stock solution of gemcitabine was diluted in water and had a concentration of 50mM and was stored at 4°C.

2.3.1 Generation of Suit-2G cells lines

Suit-2 cell lines were cultured routinely and gemcitabine was gradually added to the culture medium. The 50nM stock was diluted to lower concentrations and added to the culture medium of the flask. Initially began at 5nM and progressively increased until a concentration of 500μM was achieved. Cells were regularly observed for signs of growth and overwhelming cell death and sub-culturing was performed when cells were 80-90% confluent or contained significant cellular debris. Cell splitting was performed at longer intervals compared to parent Suit-2 cells in the initial phase but once gemcitabine was at a higher dose, there was faster and more normal passage times. The Suit-2G+ cell line was maintained at a concentration of 500μM of gemcitabine whereas a separate cell line was developed, Suit-2G- which was sub-cultured without gemcitabine. Approximately ten passages were carried out prior to any ongoing experimentation at the first instance.

2.3.2 Generation of Suit-2R and Panc-R cells lines

The Suit-2R and Panc-R cell lines were developed through a clonal isolation technique which involved plating into 96-well flat bottom plates. This is demonstrated in figure 4. Each well was filled with 100μL of stock RPMI culture medium except for A1, which was left empty. Suit-2 and Panc-1 cells were taken at a concentration of $2x10^4$ cells per millilitre and 200μL of this solution was placed into the A1 well. Then a double dilution series was performed, firstly vertically then horizontally. 100 μL was taken from the A1 cell and them placed into B1, mixing it well using a pipette. This process was repeated vertically until the H1 well and then the final 100μL discarded. Then each row was further diluted across to the 12^{th} column by adding 100μL of culture medium to column 1, mixing well and subsequently moving across diluting each well. The 12^{th} column will have 200μL after mixing and so 100μL was discarded. This dilution process means that the latter wells at the upper aspect of the plate and the former wells at the south end of the plate are likely to contain single cells. These are the wells that were closely observed as gemcitabine was added to the wells, after 24hrs allowing the cells to seed. Initially a concentration of 5nM was added to each well and the plate observed for the next two weeks. The wells that clearly contained multiple cell colonies were ignored/discarded whereas the ones with 1-2 cell colonies were observed carefully.

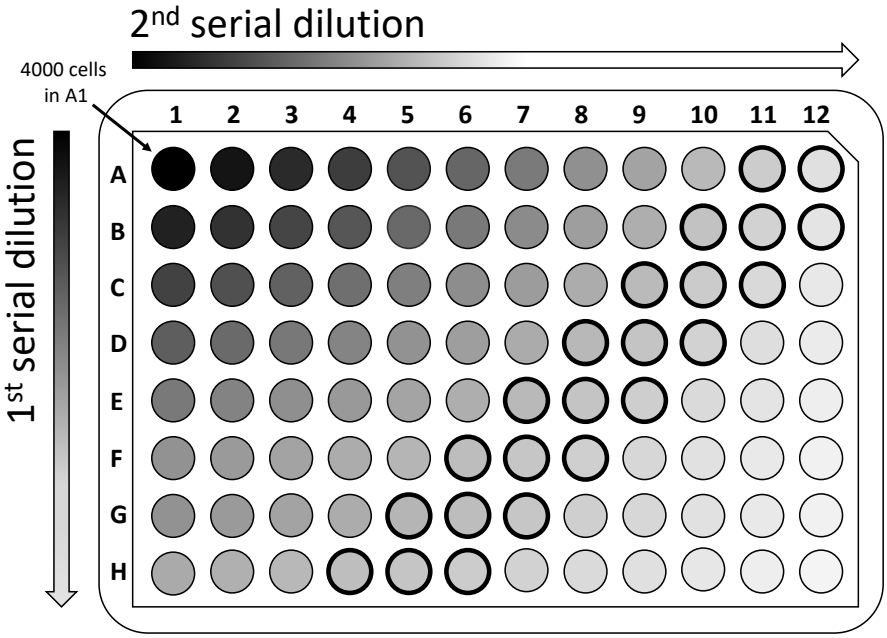


Figure 4 *Single cell dilution method on a 96-well plate demonstrating initial cell inoculation and two serial dilutions, with wells highlighted that are likely to contain one to four cell colonies*

There were attempts to add a further gemcitabine dose to the well every 4 days but this resulted in no cell growth, and the colonies were lost. Once a colony had succeeded in forming then once it had reached 80-90% confluence of the well, it was dissociated using 20µL of trypsin for a few minutes prior to adding 180µL of stock RPMI culture medium. This 200µL cell solution was then placed onto the A1 well of another 96 well plate and the double dilution process was then repeated. The dose of gemcitabine added was then doubled to 10nM and then increased initially through increments of 10nM and then by 25nM, after achieving 50nM until 250nM was reached. There were four cell colonies taken from the initial plating of Suit-2 cells and one from the Panc-1 cells that survived the step-wise increases of gemcitabine concentrations until the final dose was reached. At each step, the well that reached target confluence was the cell colony that was sub-cultured for the next step. Once the target dose of gemcitabine was reached then the next sub-culture was performed in a flat bottom six well plate without gemcitabine. The next sub-cultures were carried out using a vented T25 flask before building to a T75 flask. It was at this point that cryopreservation steps were performed to maintain a frozen stock of resistant cell lines.

2.4 Cell viability assay

General Equipment & Assays

Multiskan Microplate Photometer	(Thermo Fisher Scientific)
EZ4U – Cell Proliferation and Cytotoxicity Assay	(Biomedica Medizinprodukte GmbH)

The EZ4U non-radioactive cell proliferation and cytotoxicity assay is based upon the reduction of tetrazolium salts into coloured formazan derivatives. This assay utilises a substrate and activator reagent which once applied to viable cells, will turn the solution from straw coloured to red. The quantification of this colour change is performed using a microtitre plate reader utilising a 450nm wavelength and a 620nm reference wavelength.

Cells were plated at a concentration of $1x10^5$ per ml onto a 96 well plate at a volume of 100µL per well. They were allowed to seed overnight prior to gemcitabine treatment. The culture medium was not removed but gemcitabine added to the solution, to create a half log concentration from 0.5nM gemcitabine to 5µM. There was a control lane of cells cultured without added gemcitabine and also the outer wells were kept free from cells. Therefore, there were six rows of cells and each concentration of gemcitabine had up to six replicates but for more complex experiments and multiple cell lines, triplicates were used. The cells were cultured for 48hrs prior to performing viability assays. This was based upon preliminary standardisation experiments to assess the optimal cell densities and incubation periods to provide optimal cell viability curves. For more complex experiments involving knockdown and transfection experiments, time protocol experiments were carried out to ensure that the initial treatment parameter was given sufficient time to function prior to cell viability analysis. The EZ4U cell proliferation assay was carried out according to the manufacturer's protocol. Existing culture media was removed from each well and replaced with 200µl of fresh media and 20µl of reagent (mixture of substrate and activator) at room temperature. The plates were incubated at 37°C for 4 hours and readings were taken at 620nm and 450nm wavelengths using a microplate spectrophotometer with a shaking plate carrier. The results were analysed using Microsoft® Excel version 16.16.23 in order to standardise all cell lines to a 0-1 scale, where 0 represents the highest drug dosing and 1 is the control cell replicates. These results were then plotted as a log scale vs normalised absorbance and a drug dose sigmoidal curve fitted using GraphPad Prism version 8.42. This allowed a calculation of the half maximal inhibitory concentrations (IC50) of the cell lines and treatment parameters.

2.5 Extraction of RNA

General Equipment & Assays

RNeasy Mini Kit	(Qiagen)
EZ4U – Cell Proliferation and Cytotoxicity Assay	(Biomedica Medizinprodukte GmbH)
NanoDrop Spectrophotometer	(Thermo Fisher Scientific)

When cells reached 80-90% confluence cultured in a T75 flask, they were dissociated as above, neutralised by culture medium and then centrifuged at 1000xg for 5 minutes. After discarding the supernatant, a PBS wash was performed and then a further centrifugation process carried out. RNA preparation was performed according to the RNeasy Mini Kit protocols. Once the supernatant was discarded, 300μL of Buffer RLT was added to the cell pellet, and vortexed to ensure no cell clumps were visible. This lysate was pipetted into a QIAshredder spin column placed within a 2ml collection tube and then centrifuged at 13,300xg for two minutes. 300μL of 70% ethanol was added to the homogenized lysate and a maximum of 700μL was transferred into an RNeasy spin column, placed in a 2ml collection tube. This tube was centrifuged at 8000xg for 15 seconds and the flow through was discarded. 700μL of Buffer RW1 was added to the RNeasy spin column and a further centrifugation process was performed at 8000xg for 15 seconds. The flow through was discarded and 500μL of Buffer RPE was added to the RNeasy spin column which was once again centrifuged at 8000xg for 15 seconds to wash the spin column membrane. The flow through was discarded and another Buffer RPE step was performed but for 2 minutes to ensure all ethanol has been removed. The RNeasy spin column was removed and placed into a new collection tube and centrifuged for a minute as an extra step to remove any carry over RPE buffer. The RNeasy spin column was removed and placed into another collection tube and 50μL of RNAse free water applied to the spin column and centrifuged at 8000xg for a minute. This completed the RNA extraction and purification process and purity was assessed using a NanoDrop Spectrophotometer. A 1μL sample of extracted RNA was placed onto the pedestal and the Windows-based application software set to RNA-40 settings. A clean spectral image was ensured and the RNA quantity was documented as ng/μL. The wavelength ratios 260/280 were checked and if the readings were divergent from an accepted purity of 2 (± 0.4) then the RNA was not used for further experiments. The samples were stored at -80°C unless immediately used for cDNA preparation.

2.6 Preparation of cDNA

General Equipment & Assays

QuantiTect Reverse Transcription Kit (Qiagen)

This procedure was performed in a DNA free hood/room with specific equipment only used for this process and cleansed using nuclease free water (DNA free in this sense meaning that no purified DNA was allowed into the space). All reagents and RNA specimens were thawed on ice and each genomic DNA elimination procedure was performed using 1μg of template RNA at a calculated volume

according to the concentration, then made up to 12µL using RNase-free water and then 2µL of gDNA Wipeout Buffer was added. This was incubated at 42°C for 2 minutes. The reverse-transcriptase master mix was made up to 6µL using 1µL of Quantiscript Reverse Transcriptase, 4µL of Quantiscript RT buffer and 1µL of RT Primer mix. This was then added to the template RNA mixture making a final volume of 20µL and incubated for 15 minutes at 42°C. A final incubation procedure was performed at 95°C for 3 minutes to inactivate the reverse transcriptase. These samples were stored at -80°C prior to being used for real time PCR reactions.

2.7 Quantitative real-time polymerase chain reaction (RT-PCR) protocols

General Equipment & Assays

Light Cycler 480	(Roche Diagnostics)
SYBR Green I	(Qiagen)
Primers	(Eurofins Genomics US)

A Roche Light Cycler 480 was used for all RT-PCR reactions utilising SYBR Green I cyanine dye for DNA nucleic acid staining. A master mix of each parameter cDNA, developed as above, contained 7µL RNase-free PCR grade water, 10µL SYBR Green and 1µL primer (0.5µL forward and 0.5µL reverse), creating a 20µL volume per sample per primer. Generally, the cDNA was often diluted in a 1:10 ratio and triplicates performed per primer. Specific Light Cycler 480 96-well plates were used for the experiments and each experiment had wells with only sterile nuclease free water and a master mix without reverse transcriptase (-RT) as controls. All preparations were carried out in a DNA free room/hood with equipment utilised only for these experiments. A sealing foil was then firmly applied to the plate prior to being placed into the Light Cycler 480. Table 1 depicts the primer sequences used for all experiments and the annealing temperatures. Table 2 demonstrates the conditions required for a typical sixty cycle reaction, using a 58°C to 72°C amplification range. CyclinD1a and CyclinD1b primers were developed using Primer-BLAST from the National Centre for Biotechnology Information. Specific primers crossing exons 4-5 and exon 4 to intron 4 for each isoform. GAPDH was used as a housekeeping gene for all other parameters to be normalised to. If there was any amplification demonstrated in the water or -RT control wells, then the whole experiment was abandoned and repeated. Once melt curves were meticulously checked, the number of cycles where threshold level was achieved was recorded. Thresholds were automatically determined based on inflection point of the curve. These results were then analysed in Microsoft® Excel version 16.16.23 for MacOS, normalised to GAPDH and the $\Delta\Delta$CT method implemented. When experiments were

repeated the ΔΔCT were analysed between experimental parent controls and resistant lines using a Wilcoxon matched-pairs signed rank test using GraphPad Prism version 8 for MacOS.

Gene	Forward Sequence (5'-3')	Reverse sequence (5'-3')
RRM1	GGAGGAATTGGTGTTGCTGT	GCTGCTCTTCCTTTCCTGTG
RRM2	CCCGCTGTTTCTATGGCTTC	CCCAGTCTGCCTTCTTCTTG
RRM2B (p53R2)	GAGGCTCGCTGTTTCTATGG	ATCTGCTATCCATCGCAAGG
CDKN1A (p21)	TAGCAGCGGAACAAGGAG	AAACGGGAACCAGGACAC
Chk1	CTTTGGCTTGGCAACAGT	CCAGTCAGAATACTCCTG
CCND1 (Cyclin D1a)	CAAGTGTGACCCRGACTGC	AAAATGCTCCGGAGAGGAGG
CCND1 (Cyclin D1b)	TGAGGAGCCCCAACAACTTC	GACCTCCCAGCCAGTCAGTA
GAPDH	ATGACCACAGTCCATGCCAT	TTGAAGTCAGAGGAGACCAC

Table 1 *A list of the forward and reverse primer sequences utilised for RT-PCR*

Phase	Cycle	Duration (min)	Temperature (°C)
Pre-incubation	1	5:00	95
Amplification	60	0:10	95
		0:10	58-60
		0:10	72
Melt	1	0:05	95
		1:00	65
Cooling	1	0:10	40

Table 2 *A generic RT-PCR protocol for the Roche Light Cycler 480. For amplification phase, the annealing temperature was 58°C for RRM1 primers, 59°C for Cyclin D1a and D1a with an additional fourth step at 84°C and 60°C for RRM2, p53R2, p21, Chk1 and the housekeeping gene GAPDH*

2.8 Single Nucleotide Polymorphism Genotyping

General Equipment & Assays

DNeasy Mini Kit	(Qiagen)
Light Cycler 480	(Roche Diagnostics)
SYBR Green I	(Qiagen)
TaqMan SNP Genotyping Assay	(Eurofins Genomics US)

DNA extraction was performed on cells plated for 48 hours, seeded at $1x10^5$ density in 2ml of stock RPMI media per well of a 6 well plate. After the allotted time, the media was extracted and two 2ml PBS washes performed per well of the plate. A cell scraper was then used to manually dissociate the cells from the base of the plate. A QIAGEN DNeasy kit was used and the initial step involved adding 400µl of Buffer AP1 and 4µl of RNase A to each well and then placing into a sterile 1.5ml collection tube. The samples were vortexed and left to incubate at 65°C for ten minutes. 130µl of

Buffer P3 were added and the samples incubated for five minutes on ice. The lysates were then centrifuged for five minutes at 20,000xg. Each sample was then pipetted into a QIAshredder spin column within a 2ml collection tube and centrifuged for two minutes at 20,000xg. The flow through was transferred into a new tube and the volume was increased by 1.5 using Buffer AW1 and mixed. 650µl of the solution was placed into a DNeasy Mini spin column within a 2ml collection tube. This was the centrifuged again at 6000xg and the flow through discarded. The remaining solution from the previous step was also then placed into the same spin column and the centrifugation repeated. A further wash was performed using 500µl of Buffer AW2 and centrifuged at 6000xg for one minute. A final was wash performed using 500µl of Buffer AW2 and centrifuged for two minutes at 20,000xg. The DNeasy spin column was placed into a fresh 2ml collection tube and 100µl of the elution buffer, Buffer AE was added and incubated for five minutes at room temperature prior to a final centrifugation process at 6000xg for one minute. The NanoDrop 2000C spectrophotometer was used to quantify the DNA extracted, using similar protocols to RNA quantification as described above. Genotypes of all cells, including Panc-1 and the gemcitabine- resistant Panc-R, were analysed using a Thermofisher TaqMan SNP Genotyping Assay and a dual-colour hydrolysis probe (SNP ID: rs9344). This probe detected a single nucleotide change (G/A), labelled with either the FAM (G major allele) or VIC (A minor allele) fluorophore. Duplicate or triplicate assays were performed for each cell line and all preparations were carried out in a DNA free room/hood with equipment utilised only for these experiments. Allele-specific polymerase chain reaction (AS-PCR) was carried out using 10µl LC480 SYBR Green, 2µl probe, 100ng DNA and variable DNase-free PCR grade H2O to give a 20µl volume per reaction. A sealing foil was firmly applied to the plate prior to it being placed into the Light Cycler 480 and probe specific conditions were applied (Table 3).

Phase	Cycle	Duration (min)	Temperature (°C)
Pre-incubation	1	10:00	95
Amplification	45	0:10	92
		1:30	60
		0:03	72
Cooling	1	0:10	40

Table 3 *A generic RT-PCR protocol for the Roche Light Cycler 480. For the amplification phase, the annealing temperature of the phase was 58°C for RRM1 primers, 59°C for Cyclin D1a and D1a with an additional fourth step at 84°C and 60°C for RRM2, p53R2, p21, Chk1 and the housekeeping gene GAPDH*

2.9 Extraction of protein

General Equipment & Assays

RIPA Lysis and Extraction Buffer	(Thermo Fisher Scientific)
Protease and Phosphatase Inhibitor Cocktail	(Thermo Fisher Scientific)
Pierce™ BCA Protein Assay Kit	(Thermo Fisher Scientific)

When cells reached 80-90% confluence cultured in a T75 flask, they were dissociated as above, neutralised by culture medium and then centrifuged at 1000xg for 5 minutes. After discarding the supernatant, a PBS wash was performed and then a further centrifugation process carried out. A lysis buffer was created using radioimmunoprecipitation assay buffer (RIPA) and protease and phosphatase inhibitors at a ratio of 1:99. Once the supernatant was discarded the cell pellet was treated with 100-200µL of lysis buffer, depending on the size of the pellet. Each pellet was incubated for 5 minutes at room temperature prior to undergoing sonication for 5-10 seconds at 30% output with a continuous pulse. The treated cell pellet was then kept on ice and centrifuged at 4^{o}C at 20,000xg for ten minutes. The supernatant was collected and stored at 4^{o}C once protein concentrations were calculated. The Pierce Bicinchonic Acid assay (BCA) concentration standards were placed onto a 96 well plate at a volume of 25µL. Protein samples were diluted at a ratio of 1:10 and duplicated to ensure accuracy. 200µL of a supplied working reagent was added to each well and the plate was placed on a shaker for 30 seconds. A 30-minute incubation period was undertaken at 37^{o}C prior to absorbance measurements at 562nm on a plate reader. The protein concentrations were used to create a standard curve on SigmaPlot version 14 and each sample parameter was plotted in order to calculate protein concentrations in µg/ml.

2.10 Western blot analysis

Equipment & Reagents

Tween-20	(Sigma-Aldrich)
Mini-Protean TGX gels	(Bio-Rad Laboratories)
Laemmli	(Bio-Rad Laboratories)
β-mercaptoethanol	(Sigma-Aldrich)
Precision Plus Protein Dual Colour	(Bio-Rad Laboratories)
PageRuler Prestained Protein Ladder	(Thermo Fisher Scientific)
Trans-Blot Turbo Transfer System	(Bio-Rad Laboratories)
Trans-Blot Turbo Mini 0.2 µm PVDF Transfer Packs	(Bio-Rad Laboratories)
Clarity Western ECL Substrate	(Bio-Rad Laboratories)
ChemiDoc Imaging System	(Bio-Rad Laboratories)

Buffers

Phosphate buffered saline with Tween (PBST)

Dissolve 10 x PBS tablets into 1 L d.H20, add 1 mL Tween20 (0.1% in final solution)

Loading buffer (4X)

Lamelli solution and 10X β-mercaptoethanol, made up to appropriate volumes for number of gels and volume of columns

SDS-PAGE Running Buffer (10x):

30.3 g Tris base, 144.0 g Glycine, 50 mL 20% SDS, 950 mL d.H20

Blocking solution

5% Dried milk in PBST solution

Stripping buffer

20ml SDS 10%, 12.5ml TRIS HCl pH 6.8 0.5M, 67.5ml ultrapure water & 0.8ml β-mercaptoethanol

Western blotting was performed using ready-made 10 or 15 column any kD Mini-PROTEAN TGX gels from Biorad. 20µg of each protein sample was calculated in microlitres to be used for one column on a gel and a loading buffer composed of β-mercaptoethanol and Laemmli buffer (1:10 ratio) applied at a ratio of 1:4. Adding sterile water increased the volume of the mixture to 15µl. For multiple antibody gels a stock working solution of protein was developed. A protein ladder was always used for every experiment. The samples were heated to 90°C for 15 minutes and loaded into the gels described as above. The gels were placed into an electrophoresis tank using a running buffer diluted with water. The gels were electrophoresed at 300V for 13 minutes. The gels were then transferred to a nitrocellulose membrane using a Bio-Rad Trans-Blot Turbo Transfer System at standard settings for the particular molecular weight gels. Once transference was completed the membranes were removed and placed in a blocking solution for 4 hours. Primary antibody treatment was performed overnight at 4^{o}C and all membranes were placed on a rocker. The primary antibodies are listed in Table 4 and the concentrations used diluted with blocking solution. The membranes were washed in PBST for five-minute intervals when the PBST solution was replaced for a total of six washes. The membranes were then treated with secondary antibodies specific to the primary antibody animal type for one hour at room temperature. Following another six-wash cycle with PBST, each membrane was probed for 5 minutes with an enhanced chemiluminescence (ECL) substrate containing peroxidase. Membranes were imaged using the ChemiDoc MP system to detect the luminescence of the specifically targeted secondary antibodies. These were performed at standard settings and

subsequent repeat procedures utilising optimal cumulative exposures for appropriate time settings. These membranes were then stripped using a stripping buffer at 38°C for 15 minutes. A re-probing process was undertaken to check consistent loading levels of the housekeeping protein β-actin. After three membrane washes with PBST, the primary antibody was applied for thirty minutes at room temperature, followed by three further PBST washes and probing with the secondary antibody for a further thirty minutes. Further images were carried out using the ChemiDoc MP system. The actual images were analysed using the Bio-Rad Image Lab 6.01 software where the ladder images were incorporated into the optimal western blots. The software allowed densitometry analysis of the original images and it was possible to normalise all results to the respective actin levels. The images presented are the whole ladder-incorporated images and bar graphs of the normalised densitometries. Multiple densitometries were compared with parent and resistant cells lines using a paired t-test using the program GraphPad Prism version 8 for MacOS.

Antibody	Company (code)	Animal	Concentration
RRM1	Proteintech (10526-1-AP)	Rabbit polyclonal	1:1000
RRM2	Abcam (ab57653)	Mouse monoclonal	1:1000
RRM2B (p53R2)	Abcam (ab8105)	Rabbit polyclonal	1:2000
CDKN1A (p21)	Cell Signal Tech (12D1)	Rabbit polyclonal	1:1000
Chk1	Cell Signal Tech (2360S)	Rabbit polyclonal	1:1000
CCND1 (Cyclin D1)	Santa Cruz (H-295)	Rabbit polyclonal	1:1000
ß-Actin	Sigma Aldrich (A5441)	Mouse monoclonal	1:20000

Table 4 *A list of antibodies used in western blotting with native animal and concentrations. All secondary antibodies were tailored to the primary animal at a 1:3000 concentration*

2.11 RNA interference experiments

Equipment & Reagents

Various siRNA	(Dharmacon Inc.)
Sterile RNAse free water	(Qiagen)
Lipofectamine 2000	(Thermo Fisher Scientific)

Gene knockdown studies were performed in order to determine effects on gemcitabine resistance, through MTS assays. Optimisation and time course studies were performed in order to assess degree of knockdown via western analysis. These were performed by utilising sterile 6-well plates and cells were seeded at $1x10^5$ density in 2ml of stock RPMI media. After 24 hours each well was treated with small interfering RNA (siRNA): RRM1, RISC-FREE and Off-Target control and a lipofectamine only control. The siRNA that was obtained was suspended in sterile RNAse free water to produce a 20µM

stock. An RNA interference regimen for a single well of a 6-well plate consists of 3µl Lipofectamine 2000 combined with 200µl of reduced serum Opti-MEM media incubated at room temperature for five minutes. 3µl of the specific siRNA was added to the media and flicked in order to mix the reagents, and then incubated at 37^{o}C for thirty minutes. This mixture was then added to the seeded cell well, drop by drop swirling the plate. A time course experiment was performed at 24, 48, and 72hrs to ensure competent knockdown of the required proteins and satisfactory control parameters. After the allotted time course, the media was stripped and washed with 2ml of PBS and the cells were either scraped using a cell scraper or dissociated using trypsin prior to following the protein extraction protocols and western analyses.

A similar protocol was performed for a knockdown study on a 96 well plate. Cells were seeded as per previous MTS studies (as above) and an additional 24hr time period was allowed for siRNA treatment. Adjustments to the knockdown regimen were made based upon 96-well surface growth area. Stock regimens consisted of 10µl siRNA, 10µl of Lipofectamine 2000 and 650µl of Opti-MEM and 7µl was added to each well of a 96-well plate. After a 24hr time period the MTS protocol was followed as described above.

2.12 cDNA transfection experiments

Equipment & Reagents

Tranfection ready cDNA	(OriGene Technologies Inc.)
Lipofectamine 2000	(Thermo Fisher Scientific)

DNA plasmid transfection studies were performed in order to determine effects on gemcitabine resistance, through MTS assays. A 24hr time course study was performed in order to assess degree of protein levels via western analysis. These were performed by utilising sterile 6-well plates and cells were seeded at 1x10^{5} density in 2ml of stock RPMI media. Transfection ready cDNA was purchased from Origene with the RRM1 insert in a pCMV6-entry mammalian expression vector as a 10µg stock made to a 100ng/µl. A transfection protocol was used using 1-5µg of cDNA per well of a 6-well plate and 5-250ng per well of a 96-well plate. For the 24hr protein expression experiment each 6-well plate was treated with 1µg of cDNA. Prior to treatment, the transfection solution was prepared by adding the cDNA to 200µl of Opti-MEM and rested for five minutes prior to adding 3µl Lipofectamine 2000. This was incubated for thirty minutes and then added to a single well of a 6-well plate, drop by drop, swirling in the process. A lipofectamine control and empty plasmid was also used for these experiments. After the allotted time course, the media was stripped and washed with 2ml of PBS and

the cells were either scraped using a cell scraper or dissociated using trypsin prior to following protein extraction protocols and western analyses.

A similar protocol was performed for a transfection study on a 96 well plate. Cells were seeded as per previous MTS studies and an additional 24hr time period was allowed for siRNA treatment. Adjustments to the transfection regimen were made based upon 96-well surface growth area. Stock regimens consisted of 10µl cDNA, 3µl of Lipofectamine 2000 and 2ml of Opti-MEM and 10µl was added to each well of a 96-well plate. After a 24hr time period the MTS protocol was followed as described above.

2.13 Cell sorting experiments

Fluorescence activating cell sorting (FACS) experiments were performed using propidium staining and a Thermofisher Attune NxT flow cytometer. Cell lines were treated with a median IC50 dose (of all IC50s) of gemcitabine (50nM) or a high dose of gemcitabine (250nM) for 48 hours prior to fixation and staining. A repeat set of experiments were performed on all cell lines with an additional parameter of collecting all cells, including those detached and within the supernatant.

Specific Equipment and Reagents

Attune ® NxT Flow Cytometer	(Thermo Fisher Scientific)
Propidium Iodide	(Sigma-Aldrich)
RNAse A solution	(Sigma-Aldrich)

2.13.1 Cell fixation and DNA staining

All treatment parameters and cell lines were seeded at $1x10^5$ onto a 6-well plate in stock RPMI media and allowed to adhere overnight. The cells were treated for 48 hours with gemcitabine by the addition of a calculated dose of the drug. After the treatment time the culture medium was removed and the cells washed in 1ml of PBS. 100µl of trypsin was then applied to dissociate the cells and 900µl of media was used to neutralise after a few minutes. The cells were then placed into a collection tube and centrifuged at 100xg for five minutes at 4^{o}C. Where appropriate the supernatant was preserved and kept separately, and then combined with the dissociated cells and centrifuged together. Two ice cold PBS washes were performed and PBS aspirated leaving the remaining pellet. The final centrifugation was sometimes repeated in order to ensure all PBS was removed. The cells were vortexed at a low speed and cold 70% ethanol was added to the cells in a dropwise fashion. These cells were kept on ice for at least one hour to ensure fixation.

The staining process was performed using propidium iodide at a concentration of 10µg/ml. The cells were centrifuged at 200-300xg for five minutes to remove the ethanol, and a 5ml PBS wash with 0.05% Tween-20 was carried out. After discarding the supernatant, the cells were re-suspended in 0.5-1ml of PBS with 0.05% Tween-20 and 10µg/ml propidium iodide with 0.1mg/ml RNAse A. This was left at room temperature for at least an hour prior to analysis.

2.13.2 Flow Cytometry

The Thermofisher Attune NxT flow cytometer was used for cell cycle analysis incorporating the blue laser that functions at 488nm wavelength. All samples were tested at the same settings and a threshold of 10,000 events was used. Data was analysed using FCS Express 7 for MacOs, from De Novo software and each data set was analysed in the same manner to ultimately produce a histogram of count against cell area. The gating was performed on a cell area vs height scatter plot to exclude aggregates and debris. The software in-built modelling program allowed analysis of the normal distributions of G1 and G2 curves and the S-phase population. These percentages are displayed as bar graphs in the results sections.

2.14 Neutral Comet Assay

Equipment & Reagents

CellRad X-Ray Irradiator	(Precision X-Ray Inc.)
Normal melting point agarose (1 %) in dH_2O	(Thermo Fisher Scientific)
Low melting point agarose (1 %) in PBS	(Thermo Fisher Scientific)
SYBR Gold 1 in 10,000 in dH_2O, pH 8.0	(Thermo Fisher Scientific)

Buffers

Lysis Buffer

800ml dH_2O, NaCl (146.1 g, 2.5 M), EDTA disodium salt (37.2 g, 100 mM), Tris base (1.2 g, 10 mM), 1% N-lauroylsarcosine (10 g), at pH 9.5 (using NaOH (8g) and 5M NaOH)

Adjusted to 1 L and stored at 4°C. Working solution completed using 1 ml DMSO and 1 ml Triton X-100 to 98 ml cold lysis buffer

Electrophoresis Buffer (5X)

800ml dH_2O, 54 g Tris base, 27.5 g boric acid, 20 ml of 0.5 M EDTA (pH 8.0)

This was adjusted to a pH of 8.3 and a volume of 1L and stored at toom temperature.

Working solution of 1.5L consisted of 300ml of 5X buffer with 1.2L of distilled water

A neutral comet assay was performed in order to assess double strand DNA damage. Treatment parameters included low dose gemcitabine (50nM) treatment and high dose treatment (250nM) with negative controls and irradiated cells as positive controls. Slides were prepared by placing 800µl of normal melting point agarose gel, covered with a coverslip. Once set, the coverslips were removed and the slides left to dry overnight. There were two technical replicates and two biological replicates of every cell and treatment parameter. All cells were cultured and treated in the exact same way as in section 2.12.1 and dissociated and neutralised with trypsin and stock RPMI media. A 250µl volume of cells was placed onto the pre-prepared slides and 1ml of low melting point agarose gel placed atop with a coverslip. These slides were placed on ice for two minutes. For the positive control cell lines, the cell suspension was placed into a 24-well plate and irradiated at 8Gy using a CellRad X-Ray Irradiator. These were then placed on the pre-prepared slides as described above. After two minutes all slides had the coverslips removed and placed into Coplin jars containing fresh cold lysis buffer. The jars were left overnight and then washed twice in cold electrophoresis buffer. The slides were transferred to a black horizontal electrophoresis tank and covered in cold electrophoresis buffer for 30 minutes to allow DNA to unwind. The slides were then electrophoresed at 25V (~20 mA) for 25 minutes. Slides were removed and washed twice in PBS buffer for five minutes. Slides were dried overnight and then rehydrated in distilled water for thirty minutes. SYBR Gold nucleic acid stain was diluted in distilled water to give a 1X concentration and 1ml applied to each slide for thirty minutes. Excessive fluid was shaken off and slides were then allowed to dry prior to analysis. Each slide was imaged using a fluorescence microscope and ten images were taken per slide. The Komet 6.0 image analysis software (Andor technology) was used to quantify DNA of the comets. The software gives a percentage proportion of total DNA in the tail of the comet once the head and tail were manually located. Five readings per image were taken, giving fifty results. There were two biological and two technical replicates giving a total of two hundred readings per cell treatment parameter. Statistical comparison between parent and resistant cells were compared using unpaired t-test using Welch's correction with the program GraphPad Prism version 8 for MacOS. A single slide representative image is given for all treatment parameters in the results section.

3. Results

3.1 Generation of gemcitabine resistant cell lines

The development of gemcitabine resistant pancreatic cell lines was carried out using the two separate methods outlined in the Materials and Methods. The established pancreatic cancer cells lines, Suit-2 and Panc-1 were employed to generate resistant cell lines. Table 5 depicts the cells lines created: Four clonally resistant Suit-2 lines and one clonally resistant Panc-1 cell line and one adaptively resistant Suit-2 line.

During the development of the clonally resistant Suit-2 cell lines, replenishing the gemcitabine treatment by adding a further concentration of the drug to the individual wells of a 96-well plate was attempted twice a week. The single cells that underwent mitosis subsequently became necrotic and the islands of cells diminished and thus this element of the design was abandoned. Developing adaptive resistance was more straightforward; Suit-2 cells rapidly grew in T75 flasks containing culture medium with additive gemcitabine. Fifteen cell passages were performed in both Suit-2G+ and Suit-2G- prior to any experimentation to test the relevance of gemcitabine infused culture medium in Suit-2G+ compared to normal drug free media in Suit-2G-.

Parent cell line	Clonally resistant	Adaptively resistant
Suit-2	Suit-2R Suit-2R2 Suit-2R3 Suit-2R4	Suit-2G+ Suit-2G-
Panc-1	Panc-R	-

Table 5 *Depicting the types of resistant cell lines developed from the two parent immortalised pancreatic cancer cell lines*

In order to confirm resistance, MTS cell viability assays were performed on all cell lines. A drug dose response curve was calculated and the concentration of gemcitabine that induced a 50% reduction in cell viability (IC50) was calculated. Representative examples of MTS assays are demonstrated in Figures 5-7 with IC50 results in Table 6. The gemcitabine IC50 of the Suit-2 cell line is 10.1nM and the clonally resistant lines, Suit-2R to Suit-2R4 had readings of 24.3nM, 22.8nM, 86.7nM and 52.7nM respectively. The Panc-1 cell line had an IC50 of 15.4nM and the clonally resistant Panc-R IC50 was 61.7nM. The Suit-2G cell lines show a clear dependence on gemcitabine to maintain resistance. The Suit-2G+ cell line was maintained in culture medium with 500μM of gemcitabine and additional MTS experiments fail to show a decrease in viability in concentrations up to 100μM of gemcitabine. This is shown in figure 6; with no appreciable drug dose response given the normalisation process

(minimal absorbance 1.97 and maximum absorbance 2.32). Within the same figure, the Suit-G- curve closely follow the Suit-2 curve with a 2nM difference in gemcitabine IC50s. This contrasts with the Suit-2R, Suit-2R2, Suit-2R3, Suit-2R4 cell lines that are cultured in gemcitabine free medium that maintain resistance.

Cell line	IC50 (nM)
Suit-2	10.1
Suit-2R	24.3
Suit-2R2	22.8
Suit-2R3	86.7
Suit-2R4	52.7
Suit-2G+	Unrecordable
Suit-2G-	12.0
Panc-1	15.4
Panc-R	61.7

Table 6 *The IC50 of gemcitabine in parent & resistant pancreatic cancer cell lines*

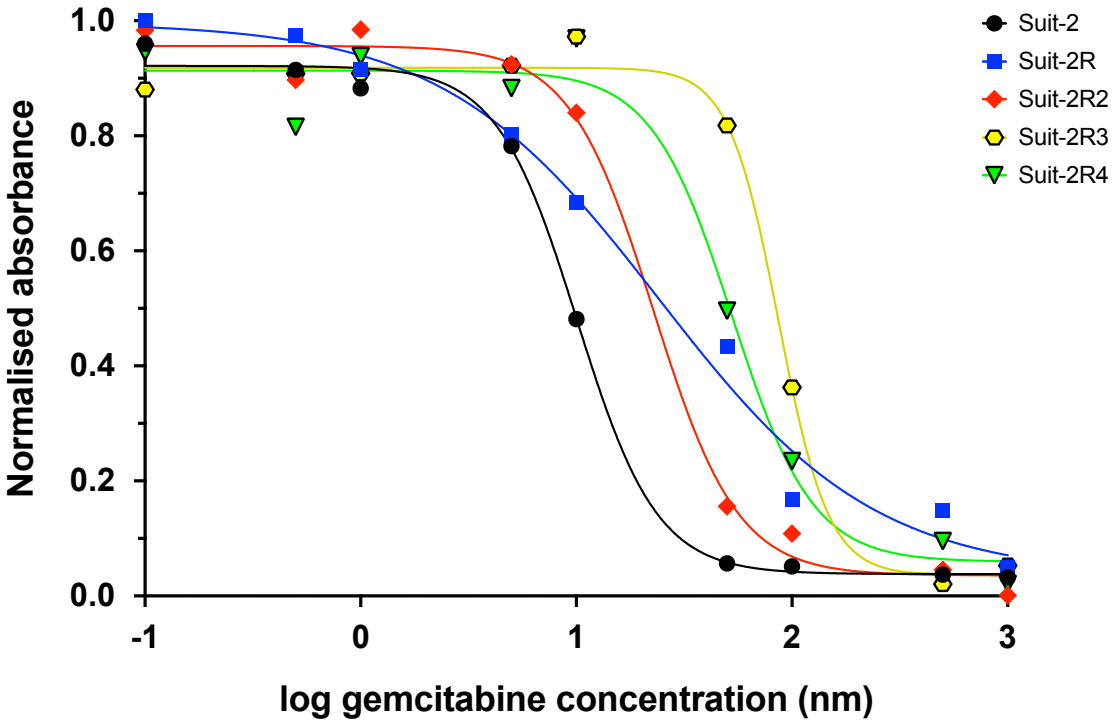


Figure 5 *A cell viability MTS assay of Suit-2 and clonal resistant cell lines demonstrating the gemcitabine resistance of each cell line. The absorbance on the y-axis was normalised to a value between zero and one to allow direct comparison of the best fit non-linear regression curve. Exact IC50 readings are shown in Table 6*

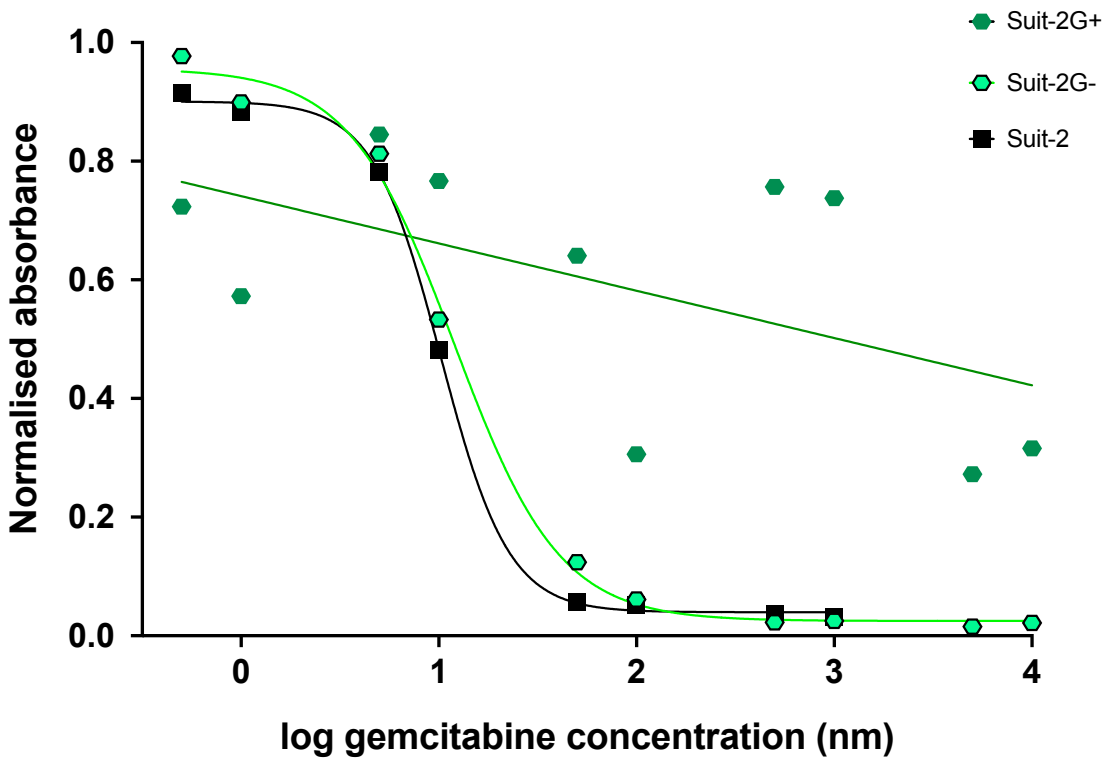


Figure 6 *A cell viability assay of Suit-2, Suit-2G and Suit-2G- to demonstrate resistance to gemcitabine. The absorbance on the y-axis was normalised to a value between zero and one to allow direct comparison of the best fit non-linear regression curve. The Suit-2G+ line does not reach a 50% reduction in viability and the linear regression model demonstrates this. Compared to the Suit-2G- best fit non-linear curve that has almost returned to the parent Suit-2 gemcitabine sensitivity. Exact IC50 readings are shown in Table 6*

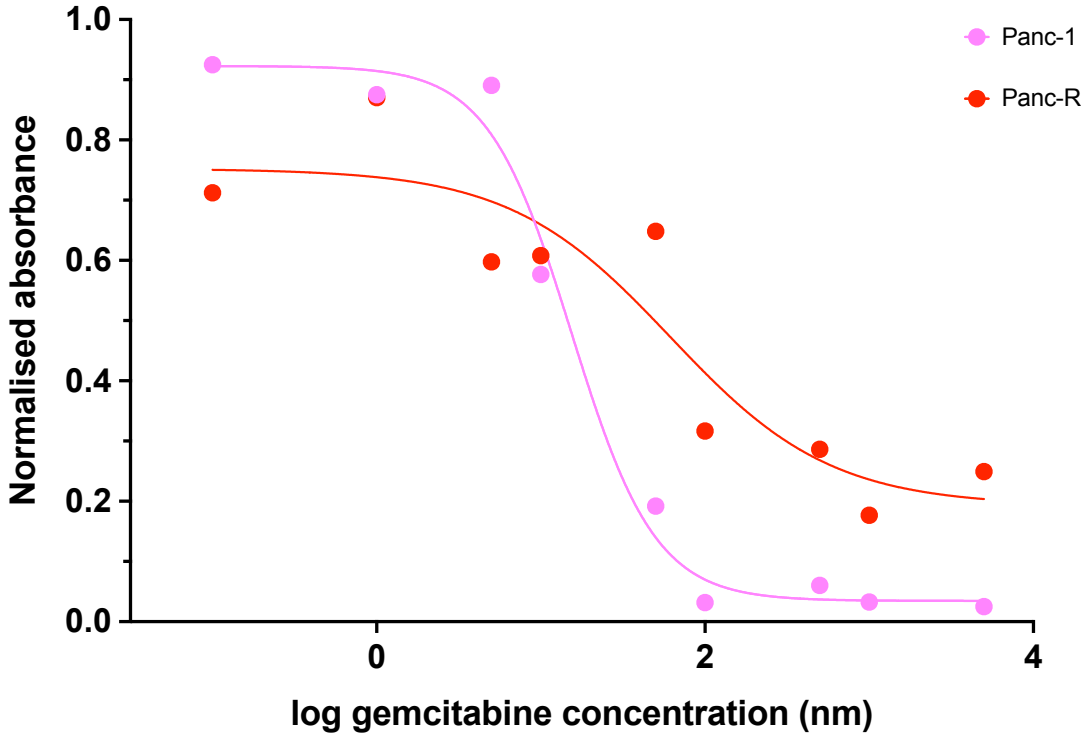


Figure 7 *A cell viability assay of Panc-1 and Panc-R to demonstrate resistance. The absorbance on the y-axis was normalised to a value between zero and one to allow direct comparison of the best fit non-linear regression curve. Exact IC50 readings are shown in Table 6*

3.2 Description of resistant cell line growth

Using the MTS assays described above, the control wells of the 96 well plate represent the standard growth of the cell lines for 72hrs. Prior to normalisation of the MTS absorbance levels, the reduction in growth rate in the Suit-2R cell line is apparent in Figure 8. It also shows similar growth rates of all other Suit-2 lines and the expected slower rate of Panc-1 cells. The resistant Panc-R line has a growth rate comparable to its parent line. In the case of Suit-2R the reduction in growth rate may be pertinent to its mechanism of gemcitabine resistance. Though this is an informal study for investigating growth rates, it highlights significant findings related to resistant cell characteristics.

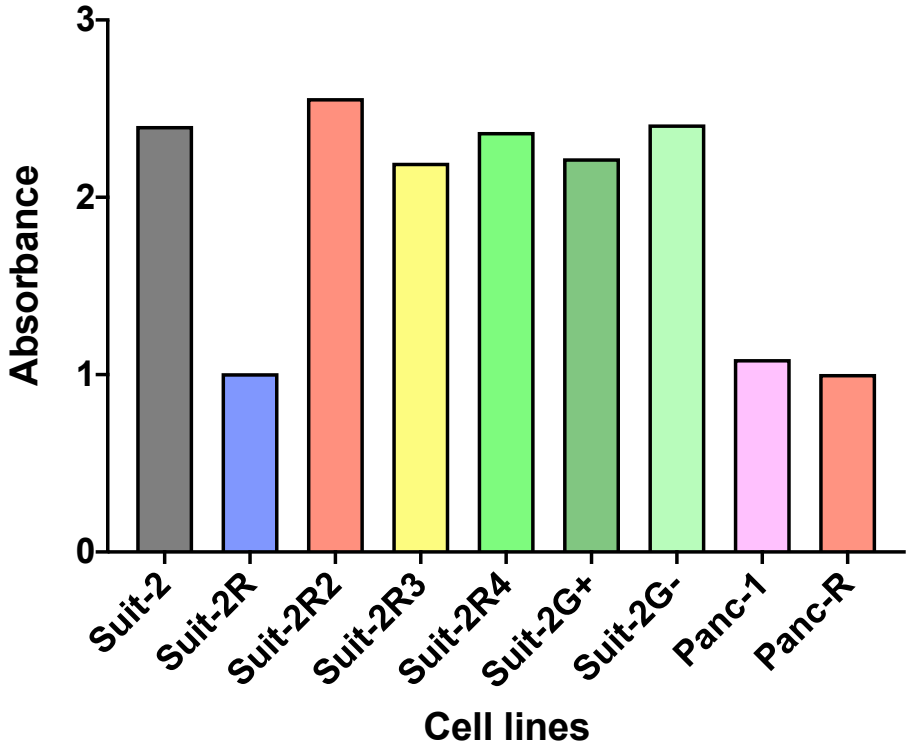


Figure 8 *A cell viability assay depicting the control parameter of the drug response assays carried out in figures 5-7. The absorbance result is a 96-hour snapshot of cell growth and demonstrates the expected lower rates of Panc-1/Panc-R cells compared to Suit-2 and the slower growth rate of Suit-2R cells compared to the parent line*

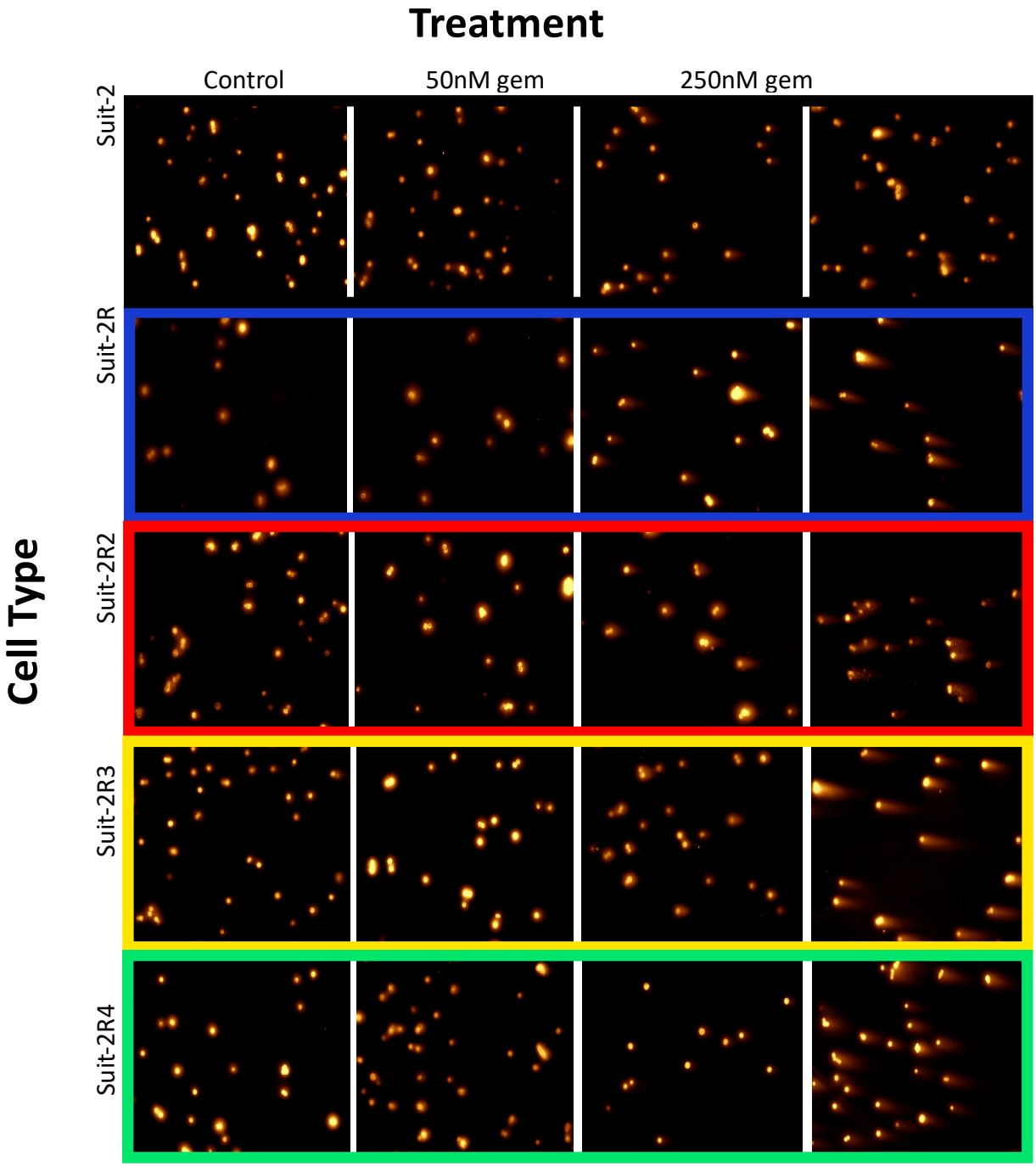
With the establishment of resistant cell lines and the slower growth rate of Suit-R, there is potentially more than a single mechanism conferring resistance to these cell lines. The effects of gemcitabine may be mitigated in order to maintain a degree of cell growth in the presence of the chemotherapy agent. Various studies have indicated that the phosphorylation process of the inactive form may play a role in sensitivity. Due to lack of consistent and reliable human equilibrative nucleoside transporter 1 (hENT1) antibody and non-significant findings in RNA expression of deoxycytidine kinase (DCK) and cytidine deaminase (CDA) across all cell lines, no further experiments on the intra-cellular activation of gemcitabine were pursued.

3.3 Investigating the cytotoxic effects of gemcitabine

It has been widely reported that gemcitabine causes a stalling of the DNA replication fork and induces double strand breaks (DSB) and the neutral comet assay quantifies the level of DSB in individual cells. Following DNA damaging conditions, cells are embedded in agarose gel on a slide, then electrophoresed in the presence of a fluorescent intercalating agent (ethidium bromide). DNA fragments migrate faster than cells and these fragments will form a 'tail' of DNA emanating from each cell. The length and intensity of the tail indicates the level of damage; another form of this assay involves denaturing of the DNA with basic conditions and so will also detect single strand breaks.

The negative drug free control arms of the experiment depict the baseline degree of DSB of the experimental process. Whereas the positive control cells are subjected to DNA damage from 8Gy radiation, a commonly used experimental and clinical dose. These afford suitable comparators to experimental parameters of gemcitabine exposure at an average of the IC50s of all resistant cell lines (50nM) and a higher dose of the drug (250nM). Figure 9 is a collection of representative images of each parameter slide and demonstrates the comets and the tail lengths of all clonally resistant Suit-2 cells. The DSB figure is a surrogate indicator of percentage DSB using the tail DNA proportion compared to the head DNA. The mean percentage of DNA DSB per treatment paradigm is documented within the table of that figure. A box and whisker plot for every experimental parameter is shown in figure 10 including cell line comparisons at 50nM and 250nM of gemcitabine treatments. Additional bar graphs are presented in order to compare the proportional increase of DSB compared to each cell line baseline. At 50nM of gemcitabine all cell lines have significantly less DSBs compared to the parent line but only Suit-2R and Suit-2R4 have significantly less damage at a dose of 250nM. Despite having less DSB DNA damage, the clonally resistant cell lines still undergo the cytotoxic effects of gemcitabine. Comparing the DSB proportion to the control of each cell, there are still DSB in all resistant clones at 50nM (figure 10h): Increases from baseline of 31% for Suit-2R and 18% for Suit-2R3 compared to 92% for the parent strain. Suit-2R2 actually had an increased percentage of double strand breaks greater than the parent (116% increase) and Suit-2R4 had a 74% increase at 50nM. Notably none of the resistant cell lines showed any resistance to DSB induced by gamma radiation (figure 10j). Indeed, all had a greater increase in DSB following gamma radiation than even the parent: Suit-2R4 having a 480% increase, Suit-2R2 and Suit-2R3 having over 400% increases and even Suit-2R having a 370% increase (compared to a 300% increase in the parent strain). This implies that in all cell lines, resistance cannot be explained specifically to DSB prevention from gemcitabine. Only Suit-2R demonstrates comparatively consistently low levels of DSB which may be due to a holistic resistance as opposed to specifically to the cytotoxic element of gemcitabine or improved

DSB repair processes. It is more apparent that the cell lines are able to mitigate the effects of DSB and tolerate them and continue to survive, especially in Suit-2R2 and Suit-2R4.



Parameter/Cell line	Suit-2	Suit-2R	Suit-2R2	Suit-2R3	Suit-2R4
Control	6.6	7.0	5.0	7.9	5.0
50nM gem	12.7	9.2	10.8	9.3	8.7
250nM gem	20.1	16.4	19.9	22.1	10.8
8Gy radiation	26.1	32.7	26.5	42.0	29.2

Figure 9 *Representative comet images are shown for Suit-2, Suit-2R, Suit-2R2, Suit-2R3 and Suit-2R4 cell lines at different doses of gemcitabine and positive and negative controls. The longer the tail of the comet, the higher the proportion of DSB in the cell. The tables provide the mean proportion of DNA in the comet tails with each treatment. Each resistant line has a lower DSB percentage at 50nM of gemcitabine but only Suit-2R4 maintains low at 250nM*

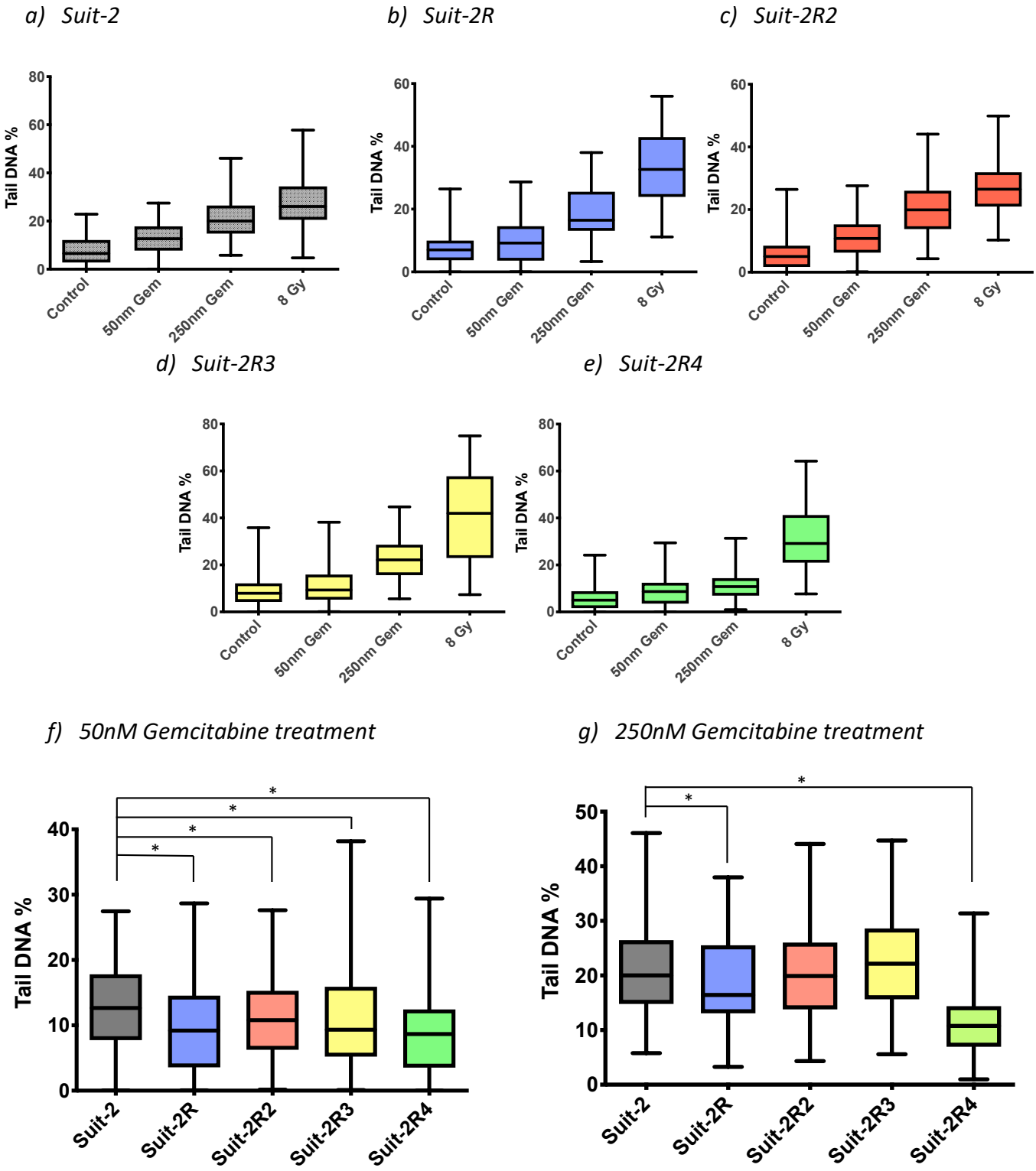
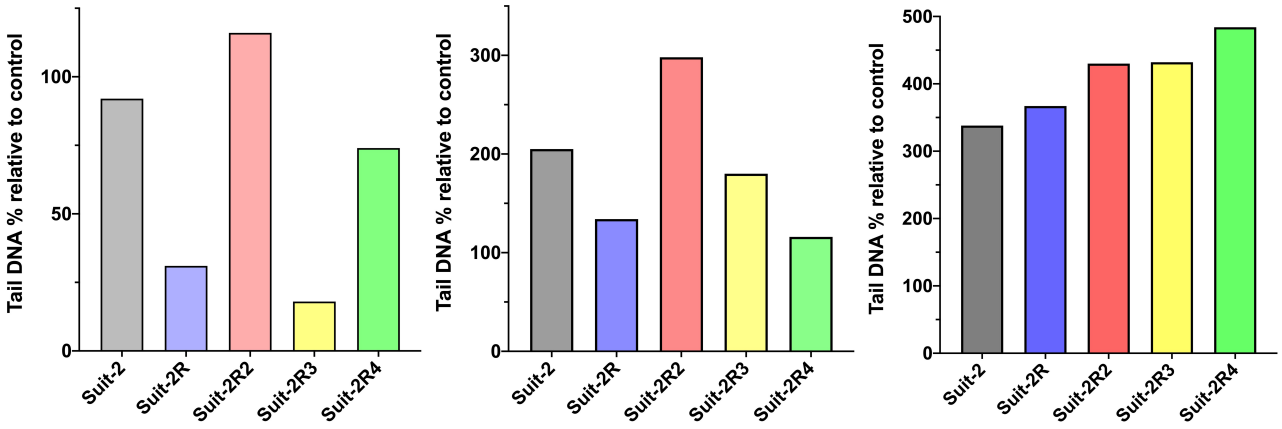


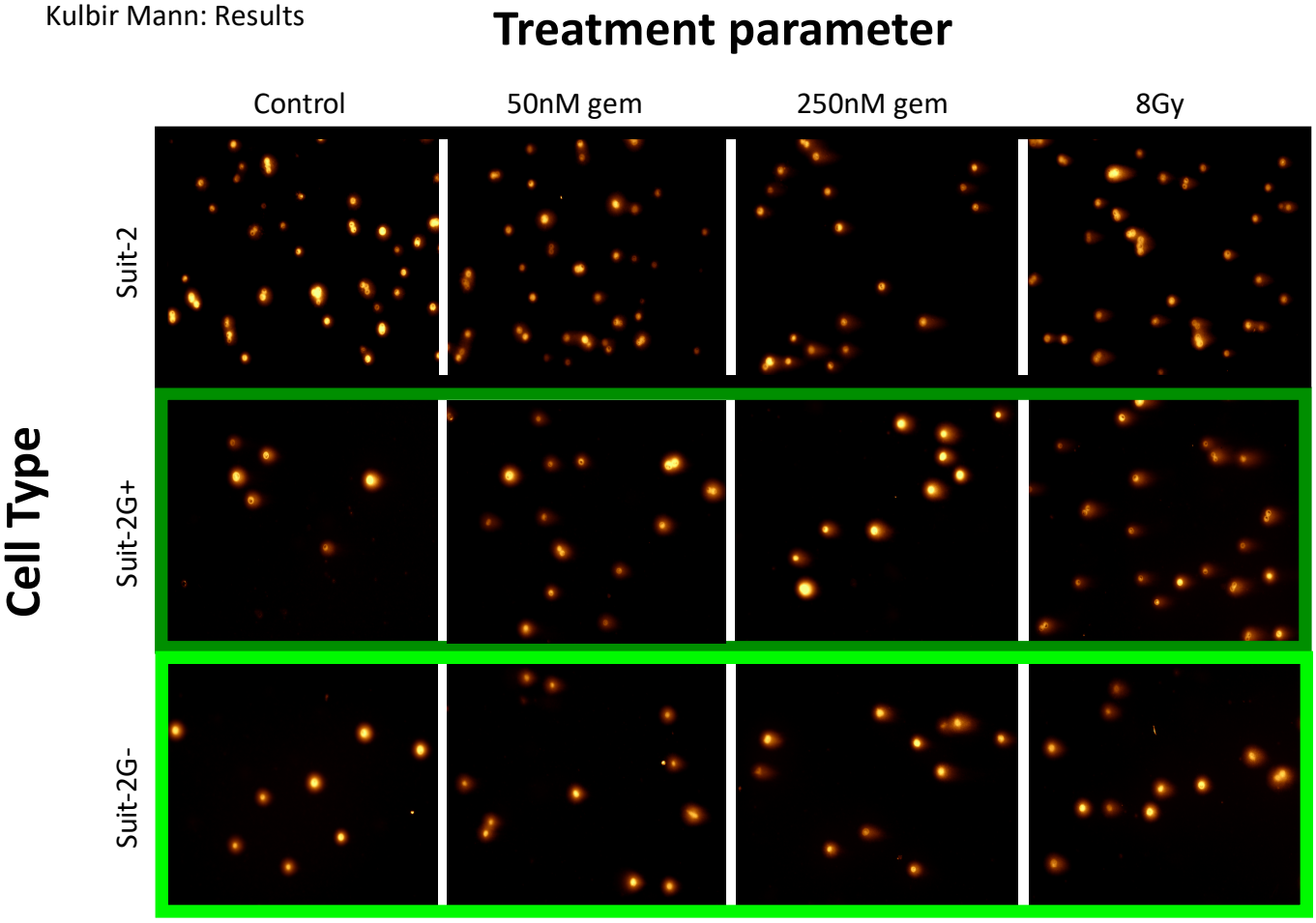
Figure 10 *Box and whisker plots to show the median, interquartile range and maximum and minimum DNA DSB percentages of Suit-2, Suit-2R, Suit-2R2, Suit-2R3 and Suit-2R4 cell lines at different treatment parameters (a-e). Figures (f) and (g) compare DSB % of each cell line at 50nM and 250 nM gemcitabine treatments, * p<0.05. This demonstrates the significantly lower DNA DSB damage at 50nM of gemcitabine in all resistant lines compared to the parent, but this is only maintained in Suit-2R and Suit-2R4 at a higher concentration of gemcitabine. Figures (h)-(j) demonstrate relative percentage change compared to control at each treatment parameter and show Suit-2R2 has higher DSB compared to even the control at 50nM and all cells sustain more damage at 8Gy compared to control. Note the y-axis have variable maximum levels.*

h) 50nM gemcitabine treatment i) 250nM gemcitabine treatment j) 8Gy radiation treatment



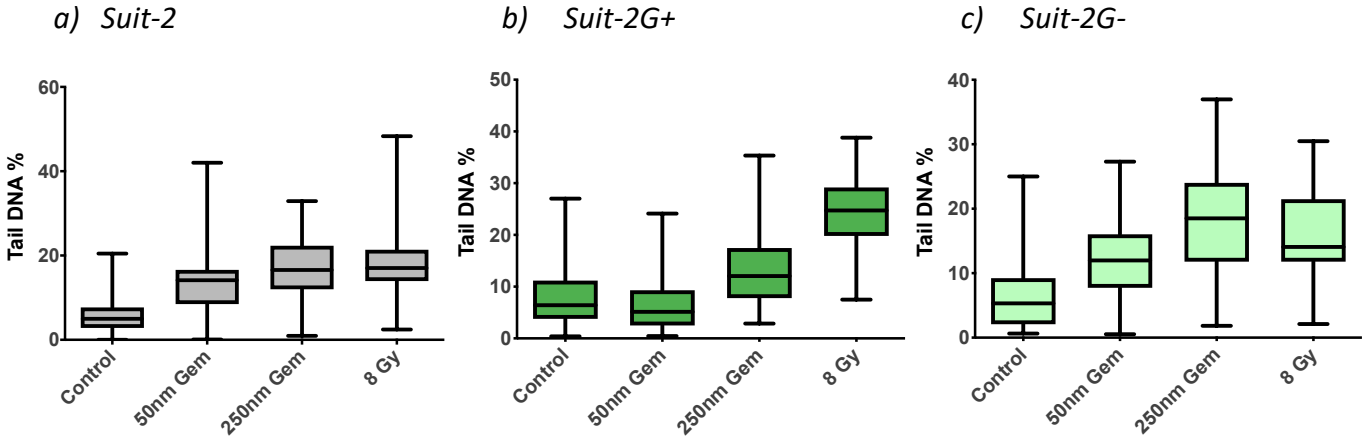
These effects are not seen clearly in the Suit-2G cell lines where figures 11 and 12 show Suit-2G+ cells have a significantly lower DSB proportion compared to Suit-2 and Suit-2G-. There is no discernible difference in percentage DSB damage between no treatment (7.8%) and 50nM gemcitabine treatment (6.6%) in Suit-2G+, but it does increase to 13.5% at the higher dose. Comparing this to Suit-2 and Suit-2G-, where an increase of 5.5% and 5.8% respectively is seen when treating with 50nM gemcitabine and 12.8% and 11.8% respectively with 250nM of gemcitabine. Figure 12f-h expand further these findings and show Suit-2G+ cells have proportionally less DSB compared to the control (-15% vs 71% at 50nM and 73 vs 164% at 250nM) and this may be explained by specific resistance to DSB or a specific response in DNA repair processing, bearing in mind that at 8Gy there are still significant proportion of double strand breaks. Suit-2G- cells are markedly different to Suit-2G+ and have increases of 89% and 181% DSB at 50nM and 250nM gemcitabine with less (148%) at 8Gy radiation. Once exposure to gemcitabine is removed, the repair processes instigated in Suit-2G+ may still persist and explain why at 8Gy it suffers the least amount of DSB in all experimental cell lines.

This further strengthens the observation that suspending gemcitabine infused culture medium returns sensitivity to gemcitabine to Suit-2G+ cells. This implies that the mechanisms to counteract gemcitabine induced replicative stress, which is still present at a lower level in Suit-2G+ cells, are evident in the Suit-2G- cell lines, but have lost resistance to gemcitabine cytotoxicity.



Parameter/Cell line	Suit-2	Suit-2G+	Suit-2G-
Control	7.8	7.8	6.5
50nM gem	13.3	6.6	12.3
250nM gem	20.6	13.5	18.3
8Gy radiation	27.8	24.3	16.1

Figure 11 *Representative comet images are shown for Suit-2, Suit-2G+ and Suit-2G- cell lines at different doses of gemcitabine and positive and negative controls. The longer the tail of the comet, the higher the proportion of DSB in the cell. The tables provide the mean proportion of DNA in the comet tails with each treatment. At both gemcitabine treatments, there is a similar DNA DSB % of Suit-2G- and Suit-2 cells, whereas it is a lower percentage in both with Suit-2G+ cells.*



d) 50nM Gemcitabine treatment *e) 250nM Gemcitabine treatment*

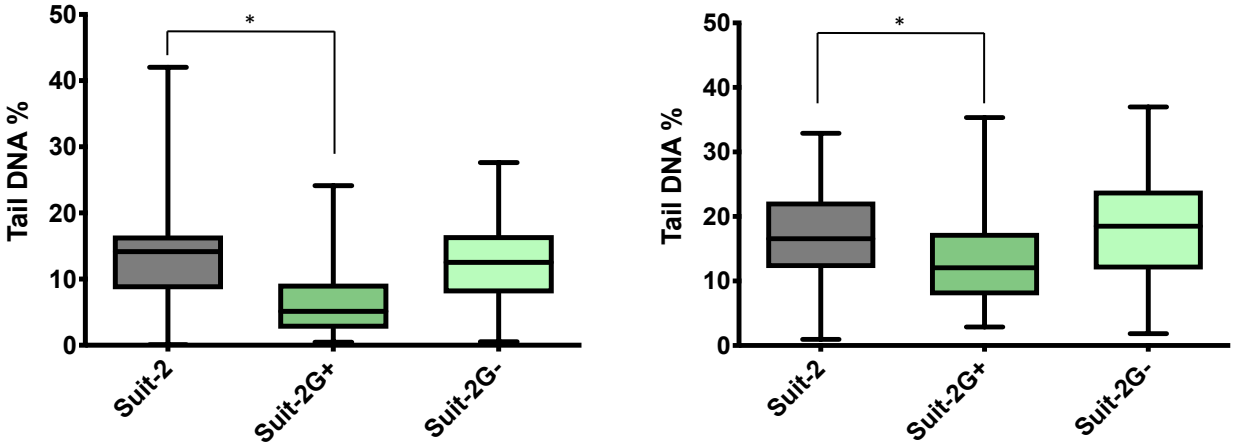
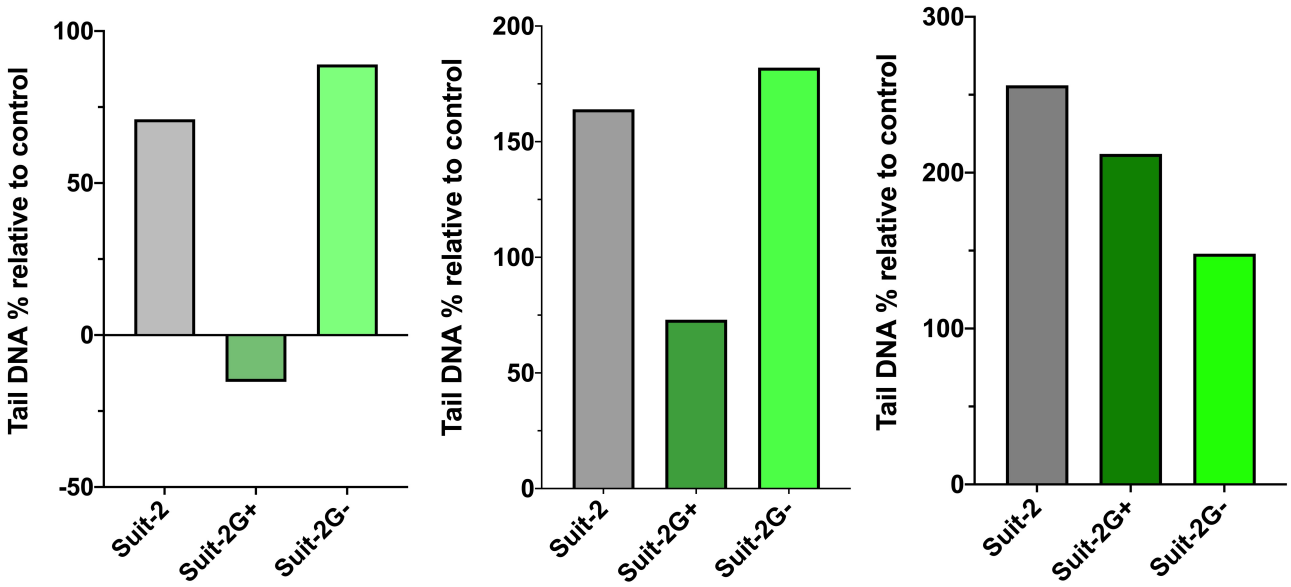


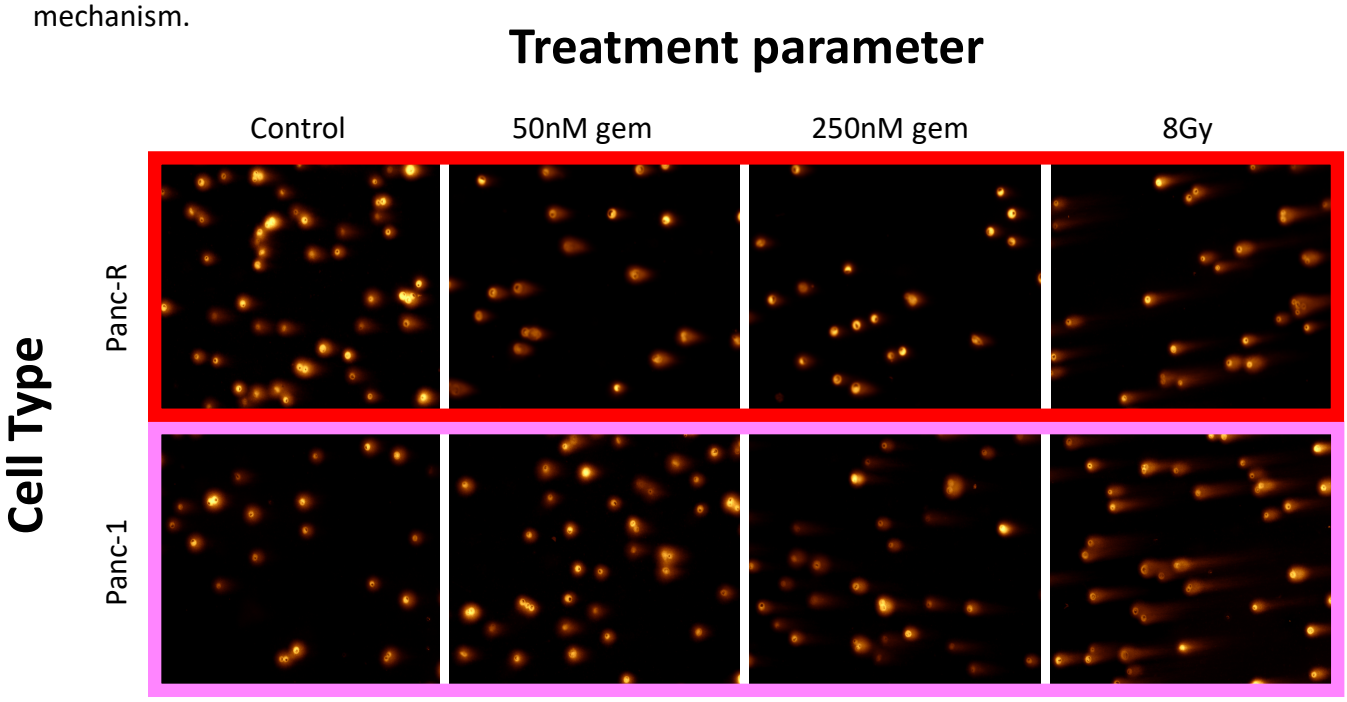
Figure 12 *Box and whisker plots to show the median, interquartile range and maximum and minimum DNA DSB percentages of Suit-2, Suit-2G+, Suit-2G- cell lines at different treatment parameters (a-c). Figures (d) and (e) compare DSB % of each cell line at 50nM and 250nM gemcitabine treatments, * p<0.05. This demonstrates the significantly lower DNA DSB damage at 50nM and 250nM gemcitabine treatments in Suit-2G+ cells but this effect is not shown in Suit-2G- cells. Figures (f)-(h) demonstrate relative percentage change compared to control at each treatment. Note the y-axis have variable maximum levels. Suit-2G+ has marked reduction in DSB when treated with the drug compared to Suit-2 but still has large DSB at 8Gy. Whereas Suit-2G- cells not returned to control levels*

f) 50nM gemcitabine treatment *g) 250nM gemcitabine treatment* *h) 8Gy radiation treatment*



Figures 13 and 14 show Panc-1 and Panc-R comet assays and box plots. They demonstrate the significantly lower proportion of DSB of the clonally resistant cell line, but only at a higher dose of gemcitabine. At 50nM the DNA damage is 8.6% and 7.6% in Panc-1 and Panc-R cells respectively but at 250nM there is a significant difference because the resistant line remains low at 6.8% compared to 11.5% in the parent line. When comparing to the control of each cell line, there is proportionally less DSB in the Panc-R line compared to Panc-1; 83% vs 69% at 50nM, 149% vs 51% respectively.

There are similar DSB proportions at 8Gy of approximately 350% implying that Panc-R may have specific mechanisms to overcome gemcitabine cytotoxicity but not as an overall DNA repair mechanism.



Parameter/Cell line	Panc-1	Panc-R
Control	4.7	4.5
50nM gem	8.6	7.6
250nM gem	11.5	6.8
8Gy radiation	21.0	21.7

Figure 13 *Representative comet images are shown for Panc-1 and Panc-R cell lines at different doses of gemcitabine and positive and negative controls. The longer the tail of the comet, the higher the proportion of DSB in the cell. The tables provide the mean proportion of DNA in the comet tails with each treatment. At both gemcitabine treatments, there is lower DNA DSB % in Panc-R cells compared to the parent Panc-1*

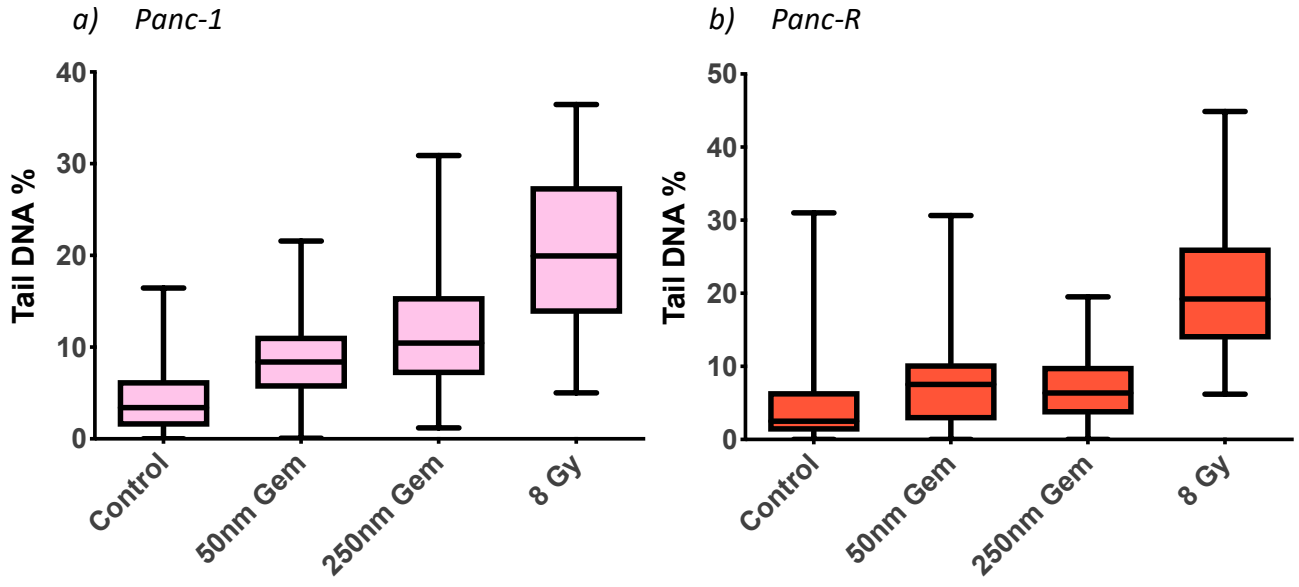
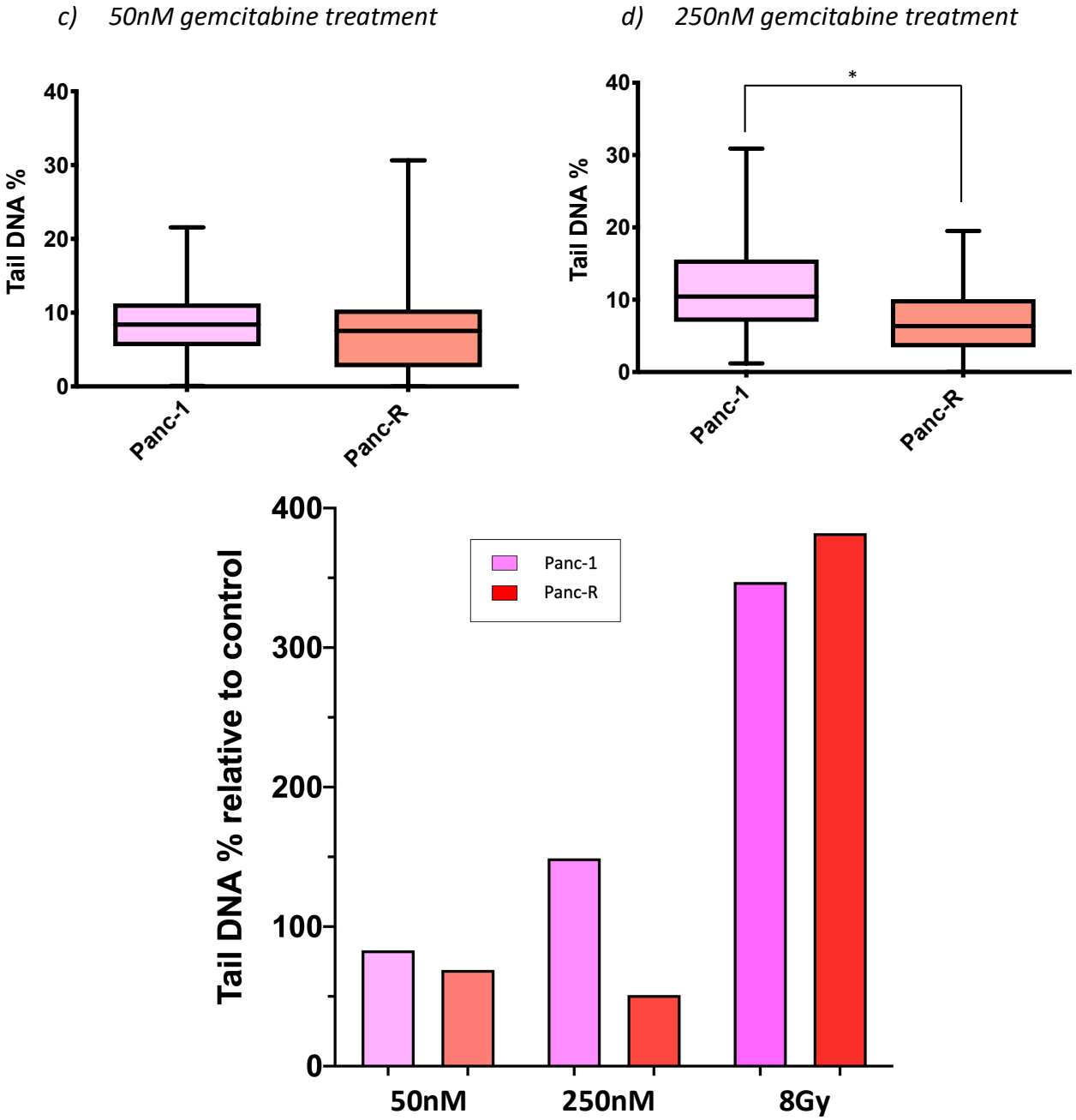


Figure 14 *Box and whisker plots to show the median, interquartile range and maximum and minimum DNA DSB percentages of Panc-1 and Panc-R cell lines at different treatment parameters (a,b). Figures (c) and (d) compare DSB % of each cell line at 50nM and 250nM gemcitabine treatments, * p<0.05. This demonstrates the significantly lower DNA DSB damage at 250nM gemcitabine treatment in Panc-R cells compared to Panc-1, this effect is not seen at the lower dose. Figure (e) demonstrates relative percentage change compared to control at each treatment parameter. The Panc-R line demonstrates proportionally similar increase in DSB compared to its control, except at 250nM where there are more DSB*



The comet assay experiments have demonstrated that DNA damage remains the cytotoxic effect of gemcitabine, albeit it at a higher concentration in the clonally resistant cell lines compared to the parent Suit-2 and Panc-1 cell lines. This would indicate that a repair mechanism has developed that may be part of the resistance process to gemcitabine but it is difficult to pinpoint whether this is

specifically to gemcitabine or an overall improvement in DSB repair functions. There is not a comparator of clonally isolated cells grown without gemcitabine, in conjunction with the resistant clones. The clonally resistant cells may overcome DSB to continue to grow whereas as Suit-2G+ may have a holistic process to improve DNA repair, given Suit-2G- has less damage at 8Gy and Panc-R may have a specific gemcitabine compensation processes. There is variation with all three groups of resistant cell lines and builds the evidence base of a complex varied set of resistance mechanisms.

DNA damage and repair are closely related to the cytostatic effect of gemcitabine. It provides an inhibitory function to ribonucleotide reductase which is responsible for the supply of deoxyribonucleotides, essential for DNA synthesis. In order to full elucidate the processes of gemcitabine resistance, the function of the ribonucleotide reductase (RR) complex and its effect on DNA repair requires establishing.

3.4 Investigating the cytostatic effects of gemcitabine

The ribonucleotide reductase complex is a heterodimeric tetramer comprising of RRM1 and RRM2 subunits with the p53 inducible p53R2 being able to pair with RRM1 in the place of RRM2. The following sections review the RNA fold change and protein levels of each of these sub-units in order to elicit the extent of the cytostatic effect of gemcitabine.

3.41 RRM1

Figure 15 shows the fold change in RNA expression of RRM1 in all cell lines. They are plotted separately because the Suit-2G+ cell line has a mean 85-fold increase in RRM1 RNA expression and obscures the graphical representation of smaller changes. This effect is not as prominent in the Suit-2G- cell lines but there is still a 14-fold increase in RRM1 RNA. There are smaller increases in the Suit-2R and Panc-R cell lines but there is a large standard deviation from the mean, inferring there is likely to be no difference. Suit-2R2, Suit-2R3 and Suit-2R4 have reduced mean RRM1 RNA expression with fold changes of 0.67, 0.36 and 0.42 respectively. Figures 16 and 17 demonstrate the impact of altered RRM1 RNA production on protein translation.

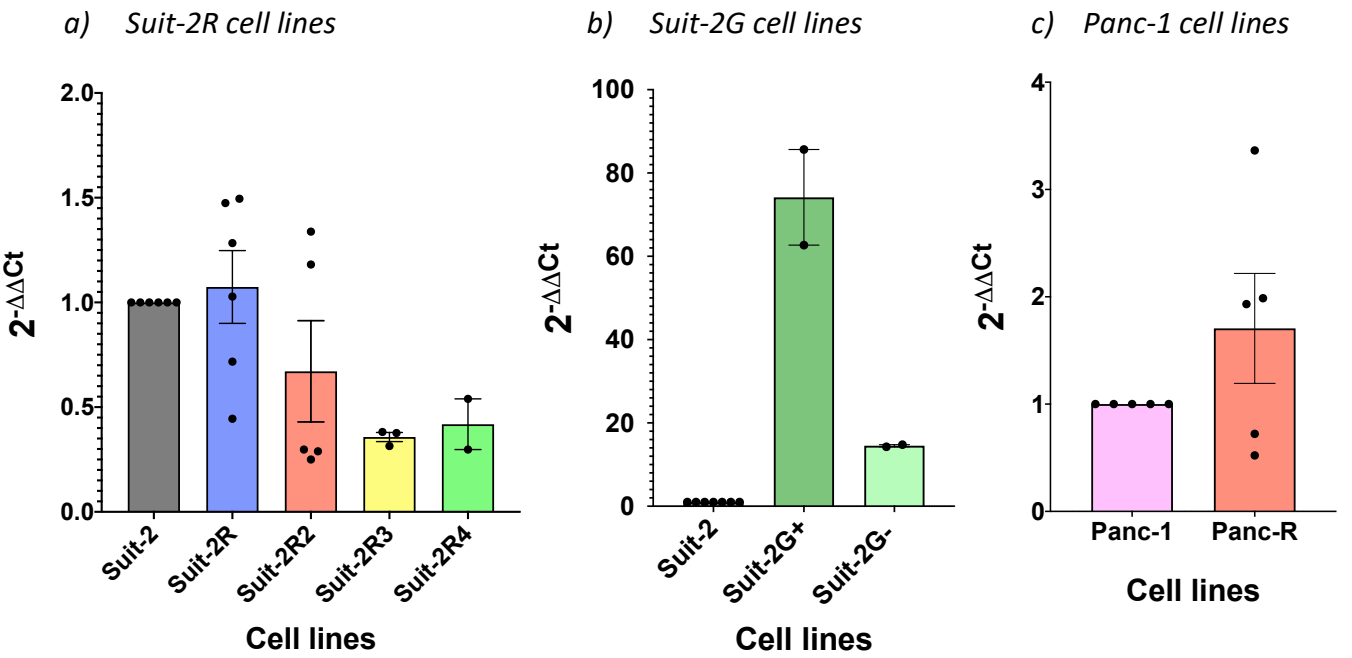


Figure 15 *Bar charts to demonstrate the mean fold change of RRM1 RNA expression in a) Suit-2 and the clonally resistant cell lines b) Suit-2 and the adaptively resistant Suit-2G cell lines c) Panc-1 and Panc-R. Each dot represents an experimental replicate and mean and standard errors are plotted for each cell lines. There is a decrease in RNA expression in Suit-2R3 and Suit-2R4 which is not seen in Suit-2R and Suit-2R2 (a). This contrasts with the significant fold change increase in the RRM1 RNA expression of Suit-2G+ cells and a smaller but persistently high increase with Suit-2G-. There is a two-fold increase in the Panc-R cell lines compared to the parent line*

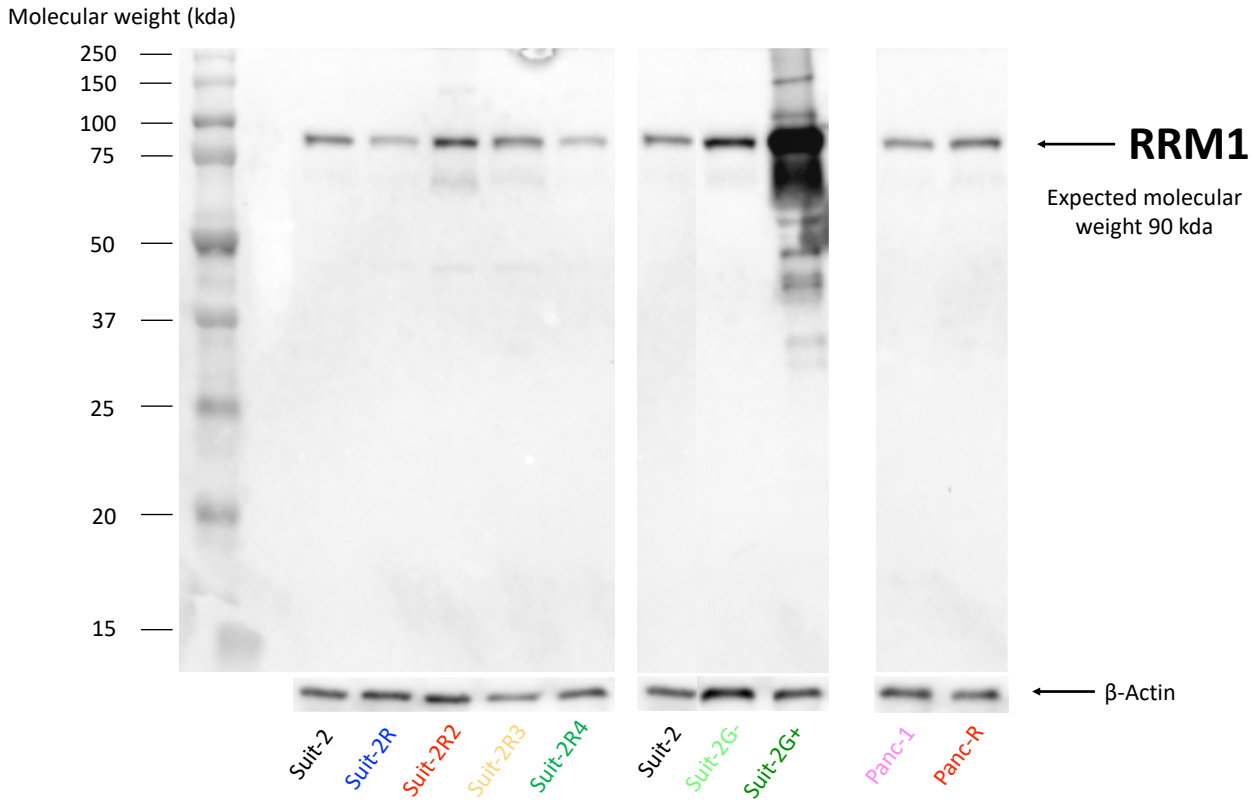


Figure 16 *A representative western blot to show the expression of the RRM1 protein at the expected molecular weight of 90kDa, with the housekeeping protein β-actin at the base of the blot. Suit-2R and Suit-2R4 show reduced protein RRM1 expression where Suit-2R2 and Suit-2R3 show the converse. The Suit-2G+ has an overwhelming increase in RRM1 expression and Suit-2G- has a smaller increase compared to Suit-2. This effect is also seen with Panc-R in comparison to Panc-1*

a) Suit-2 and clonally resistant cell lines

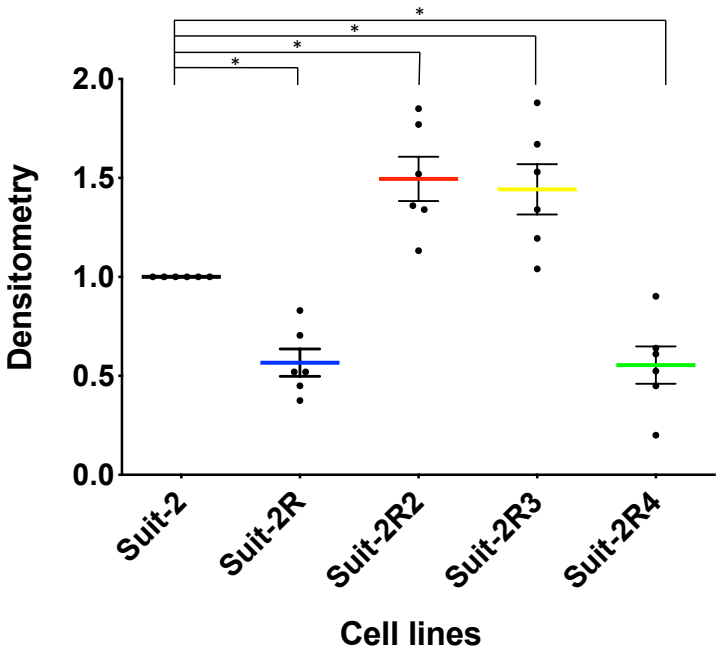


Figure 17 *A dot plot depicting the mean and standard error of every RRM1 experimental densitometry normalised to actin levels of the a) Suit-2 and Suit-2R b) Suit-2G+ and Suit-2G- c) Panc-1 and Panc-R cell lines, *<0.05. The increase in RRM1 protein levels seen in Suit-2R2 and Suit-2R4 were statistically significant as were the decreases in expression in the Suit-2R and Suit-2R4 cell lines. The marked increase in Suit-2G+ was statistically significant as was the change in RRM1 protein levels in Suit-2G- compared to the parent line. This increase was also reported with the Panc-R cell line compared to the parent Panc-1 cell line*

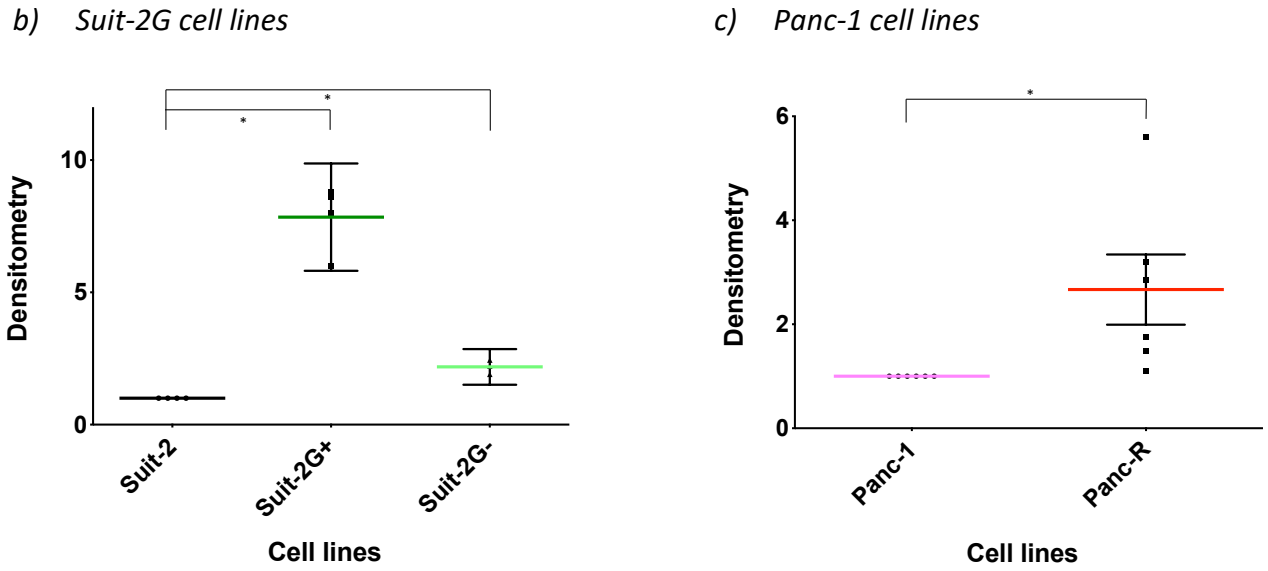


Figure 16 is a complete representative western blot of the RRM1 antibody and has been divided into sections to allow ease of comparison to parent lines, with the Suit-2 column being duplicated adjacent to the Suit-2G lines. It shows that Suit-2R and Suit-2R4 are cell lines with reduced RRM1 protein level compared to Suit-2, and Suit-2R2 and Suit-2R3 have increased RRM1 expression. This is confirmed statistically in figure 17a where all western experiments are plotted and significant differences are found in every clonally resistant line. Panc-R has a significantly higher expression of RRM1 compared to Panc-1, similar to Suit-2R2 and Suit-2R3. Suit-2G+ has an overwhelming increase in RRM1 protein level with a smaller increase in the Suit-2G- cell line compared to the parent Suit-2. These effects appear to be driven from an epigenetic level as the RNA fold changes are similarly increased as shown in figure 15b. This cannot be said about the clonally resistant Suit-2 and Panc-1 lines where figures 15a and 15c do not show significant changes in RNA expression, except that Suit-2R3 and Suit-2R4 where it is reduced. There is likely to be a post transitional modification because these cells lines have opposing RRM1 protein levels compared to Suit-2.

3.42 RRM2

The same RRM2 antibody was used throughout western analyses and in some instances two bands occurred. Although both bands were believed to be RRM2 (the faster moving band may have a modification e.g. phosphorylation) only the dominant band was included in the comparison between cell lines and conditions. Combining the bands was considered unsafe (it is unclear whether the relationship between staining intensity and concentration was the same for both) and any comparison based on the two bands would force assumptions that could not be supported experimentally. Changes to the intensity of the dominant band were chosen as measures of changes in RRM2 (which could have involved changes post-translationally or of total protein level).

Figure 18 shows the fold change in RNA expression of RRM2 in all cell lines and depicts that Suit-2R and Suit-2R4 have subtle increased in mean RNA expression, 1.2 and 2.3-fold respectively. There are decreases in mean RRM2 RNA expression in Suit-2R2, Suit-2R3 and the Panc-R lines of 0.17, 0.36 and 0.38-fold respectively. There is no appreciable change to the Suit-2G cell lines. Figures 19 and 20 demonstrate an analysis of the protein level of RRM2 and it is worth noting that there are not significant changes and after statistical analysis of all blots, only Suit-2R had reduced expression. The potential increases in RNA expression with Suit-2R, Suit-2R4, Panc-R may not translate to any substantial changes at a protein level. There may be a post translation regulation in Suit-2R cell lines but levels are still only slightly deviated from the parent cell line. It is worth noting that despite massive increases of RRM1 RNA and protein level, this does not correlate with similar RRM2 findings, the other sub-unit of the RR complex.

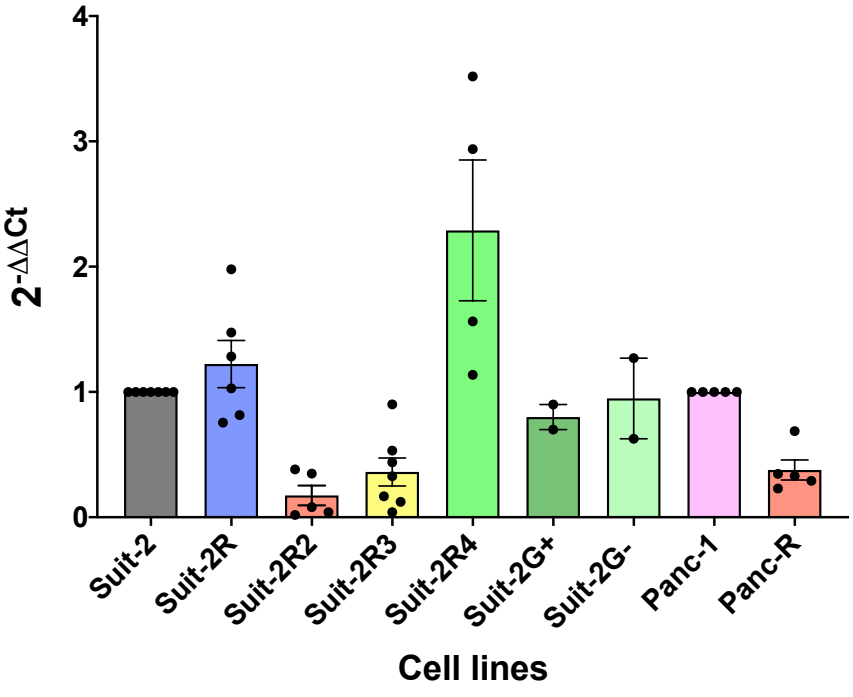


Figure 18 *Bar charts to demonstrate the mean fold change of RRM2 RNA expression in all cell lines. Each dot represents an experimental replicate and mean and standard errors are plotted for each cell lines. There is a decrease in RNA expression in Suit-2R2 and Suit-2R3 which is not seen in Suit-2R and Suit-2R4 which may have the contrary effect. No fold change is apparent to RRM2 RNA expression in the Suit-2G lines & Panc-R exhibits a reduction in RNA expression compared to Panc-1*

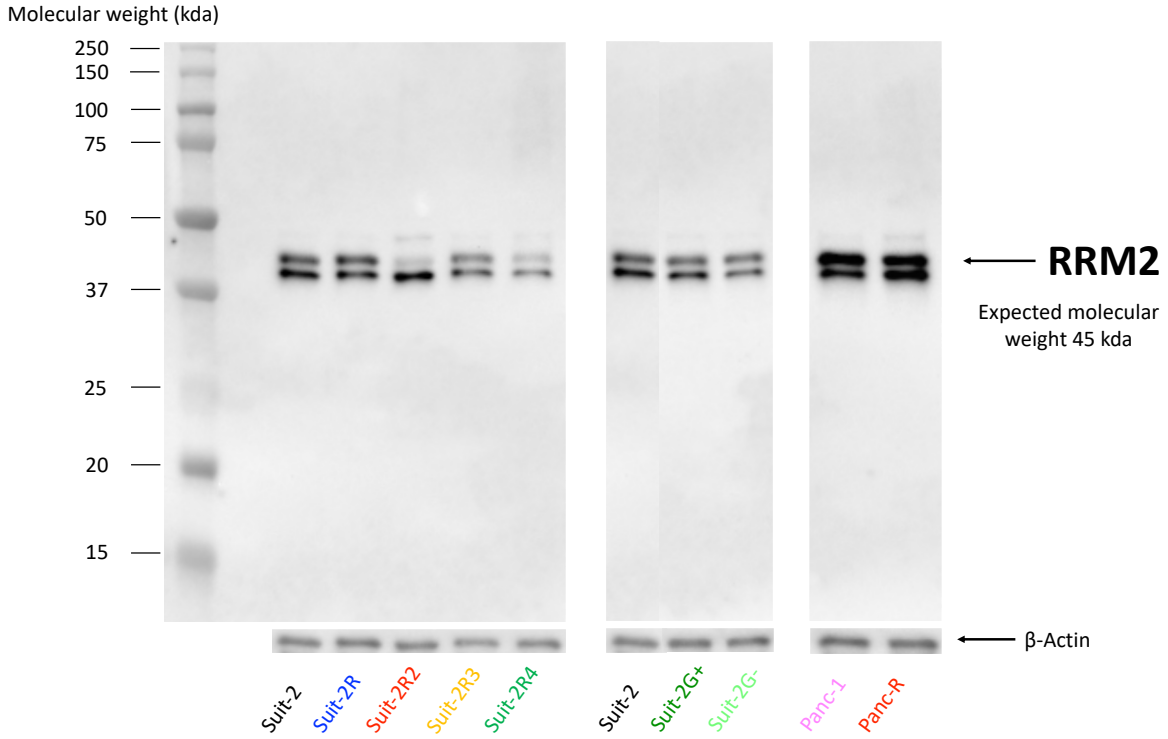


Figure 19 *A representative western blot to show the expression of the RRM2 protein at the expected molecular weight of 45kDa, with the housekeeping protein β-actin at the base of the blot. Both bands were included in the densitometry analysis. Suit-2R2 and Suit-2R4 show reduced protein RRM2 expression where all other cell lines show no change to the parent Suit-2 or Panc-1 cell line.*

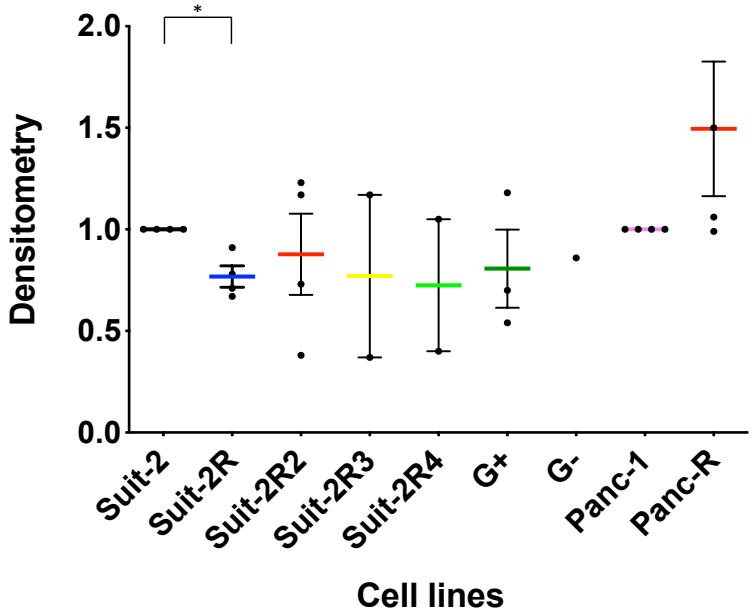


Figure 20 *A dot plot depicting the mean and standard error of every RRM2 experimental densitometry normalised to actin levels of all cell lines, *<0.05. The decrease seen in figure 19 of Suit-2R2 and Suit-2R4 were not apparent after all western densitometries were reviewed, it is worth noting that Suit-2R had significantly lower RRM2 protein level, albeit at a low level*

3.43 p53R2

The p53 inducible subunit of RR is p53R2 and figure 21 depicts the fold change in RNA expression in all cell lines. There may be similar trends in comparison to the RRM2 RNA expression. In the Suit-2 clonally resistant lines where Suit-2R2 and Suit-2R3 show a reduced expression at a mean of 0.64 and 0.61- fold change respectively. There are possible similarities with Suit-2R and Suit-2R4 with similar if not increased expressions but there is a wide error margin. With the two experimental replicates, there is a reduced mean fold change in Panc-R of 0.62 compared to Panc-1. Similar findings are found with the Suit-2G+ and Suit-2G- cell lines of 0.52 and 0.60 mean fold change.

Figures 22 and 23 show the protein level of p53R2 and there are few differences to note. Of significance (though not statistically p=0.09) is the reduction in expression in Suit-2R4 cells of almost half the level of Suit-2 cells. In comparison to other resistant cell lines, the possible reduction in RNA expression does not correlate with any significant change at a protein level. There is clearly a difference with Suit-2R4 cells which may arise at a post-transcriptional stage.

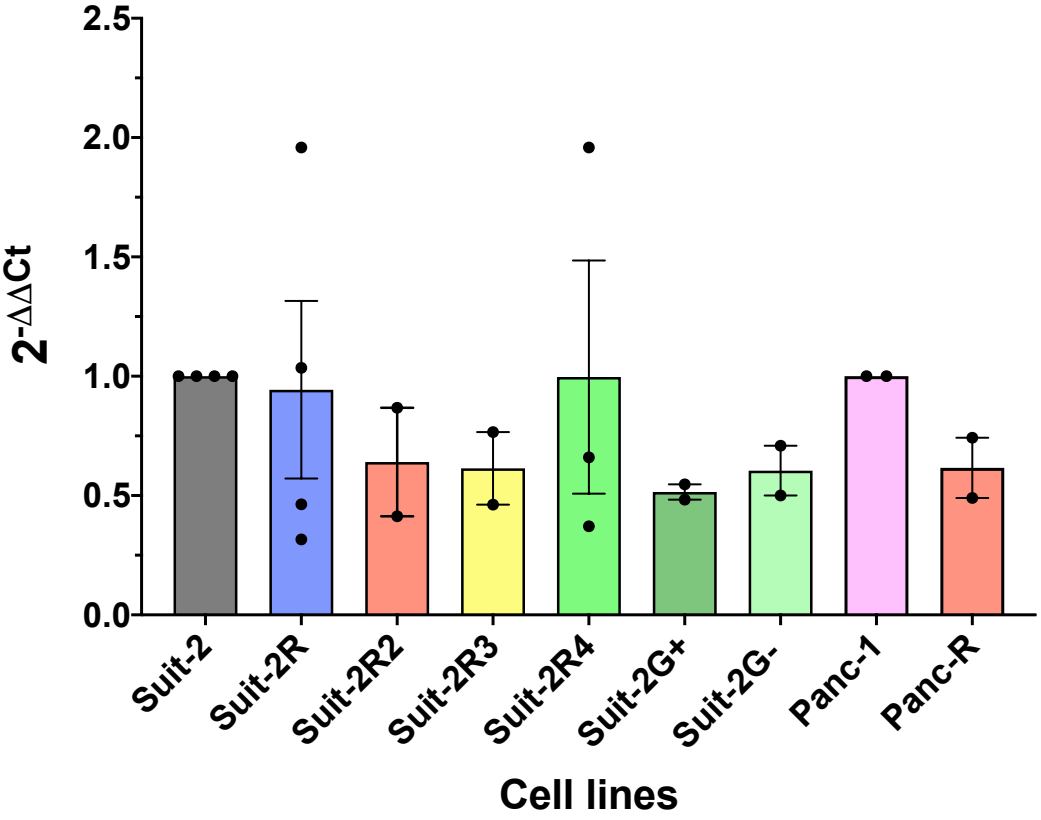


Figure 21 *Bar charts to demonstrate the mean fold change of p53R2 RNA expression in all cell lines. Each dot represents an experimental replicate and mean and standard errors are plotted for each cell lines. There is a decrease in RNA expression in Suit-2R2 and Suit-2R3, the opposite effect is potentially seen in Suit-2R and Suit-2R4, but there is a high degree of error. There are also some decreases to RNA expression in the Suit-2G lines. Panc-R exhibits a reduction in RNA expression compared to Panc-1*

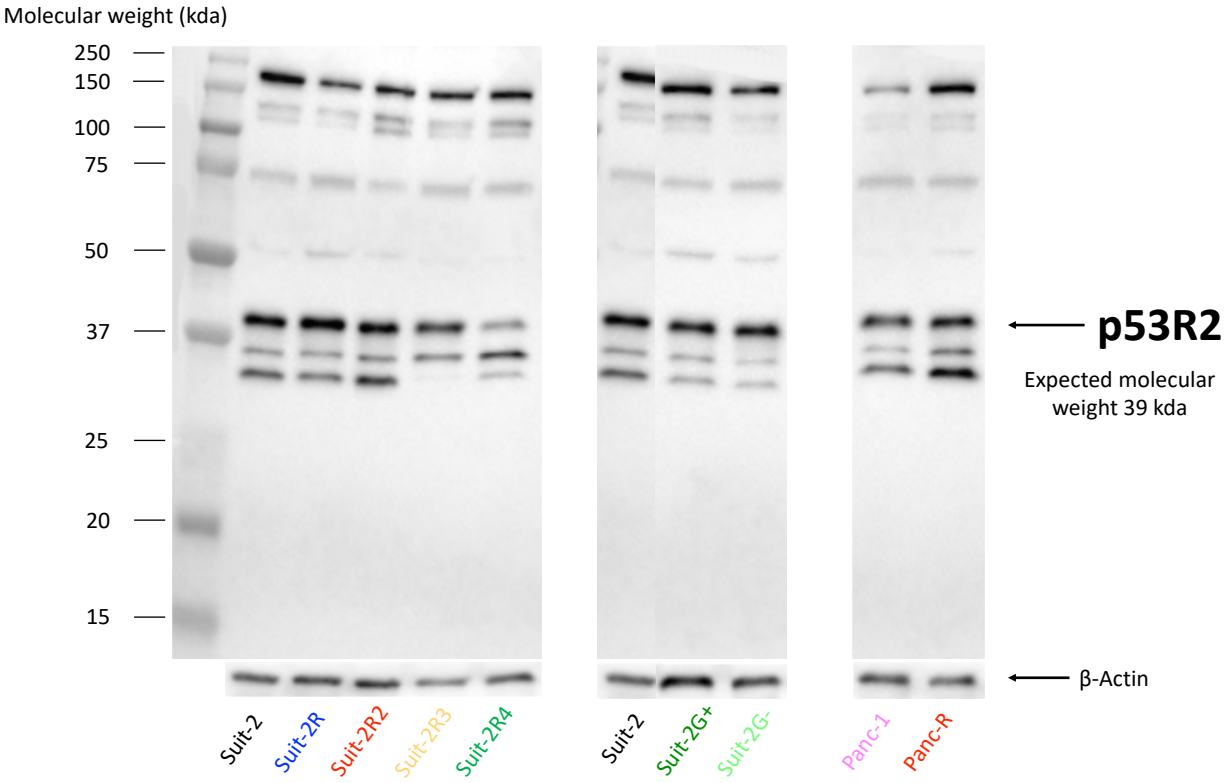


Figure 22 *A representative western blot to show the expression of the p53R2 protein at the expected molecular weight of 39kDa, with the housekeeping protein β-actin at the base of the blot. Suit-2R4 is the only cell line that exhibits any difference, a reduction in the protein level of p53R2*

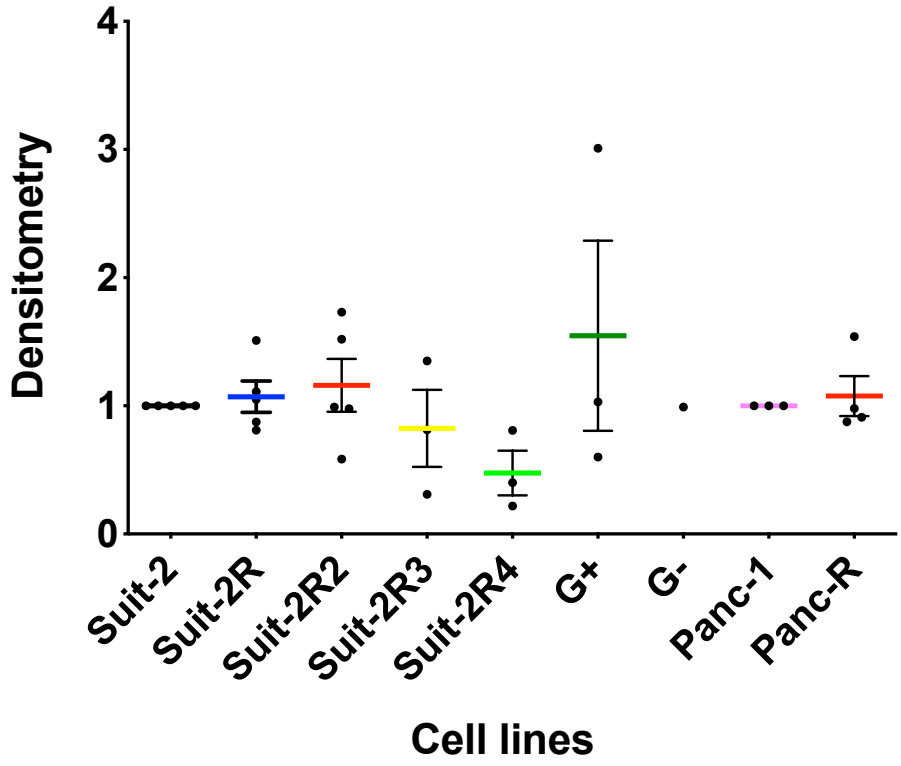


Figure 23 *A dot plot depicting the mean and standard error of every p53R2 experimental densitometry normalised to actin levels of all cell lines, *<0.05. The decrease seen in figure 22 of Suit-2R4 is apparent with a low degree of error. This did not reach statistical significance with a p-value of 0.09, but there is a clear trend to reduced p53R2 expression*

Reviewing the RR complex on the whole, RRM1 appears to play the key role in its function as there are significant changes in almost all cell lines. Whether there is reduced expression at a protein level (Suit-2R and Suit-2R4) or an increase (Suit-2R2, Suit-2R3 and Panc-R) these may arise from epigenetic modification that occur after DNA transcription. The opposite effect is seen with the adaptively resistant cell line Suit-2G+ where an 85-fold increase in RNA expression correlates directly with the massive increase in protein level, which subsequently decrease at both stages once gemcitabine stimulus is removed from cell culture (Suit-2G-).

These findings are not apparent with the small subunit of RR, even at the recruitable p53R2 level. There are some changes at translational levels, most likely reduced in Suit-2R2, Suit-2R3 and Panc-R but these do not correlate with any significant change at a protein level. Suit-2R2 and Suit-2R4 demonstrate reduced RRM2 and p53R2 protein level respectively but this does not correlate with an increase in its substitute protein.

Though the RR complex is formed from the RRM1 and either the RRM2 or p53R2 subunit, it is the larger RRM1 which may play a determining role in tampering with the cytostatic effects of gemcitabine. The following section explores this further by altering the expression of RRM1 to determine whether resistance to gemcitabine is affected.

3.5 Assessing gemcitabine resistance through RRM1 modifications

This section observes RRM1 expression in various experimental conditions. Given its low level and high-level expression in the clonally resistant cells lines, siRNA was used to perform RRM1 RNA interference to reduce expression and RRM1 plasmid transfection was performed to increase RRM1 expression. In the initial instance it was pertinent to elicit the cells response, specifically to RRM1, upon treatment with gemcitabine.

3.5.1 The effect of gemcitabine treatment on RRM1 expression

Figure 24 show western blots to observe the expression of RRM1 when the cells are treated with gemcitabine for 48hrs. The two gemcitabine doses are similar to above experiments and represent the average IC50 of the resistant cells (50nM) and a suitably high dose (250nM).

a) Suit-2 and clonally resistant lines

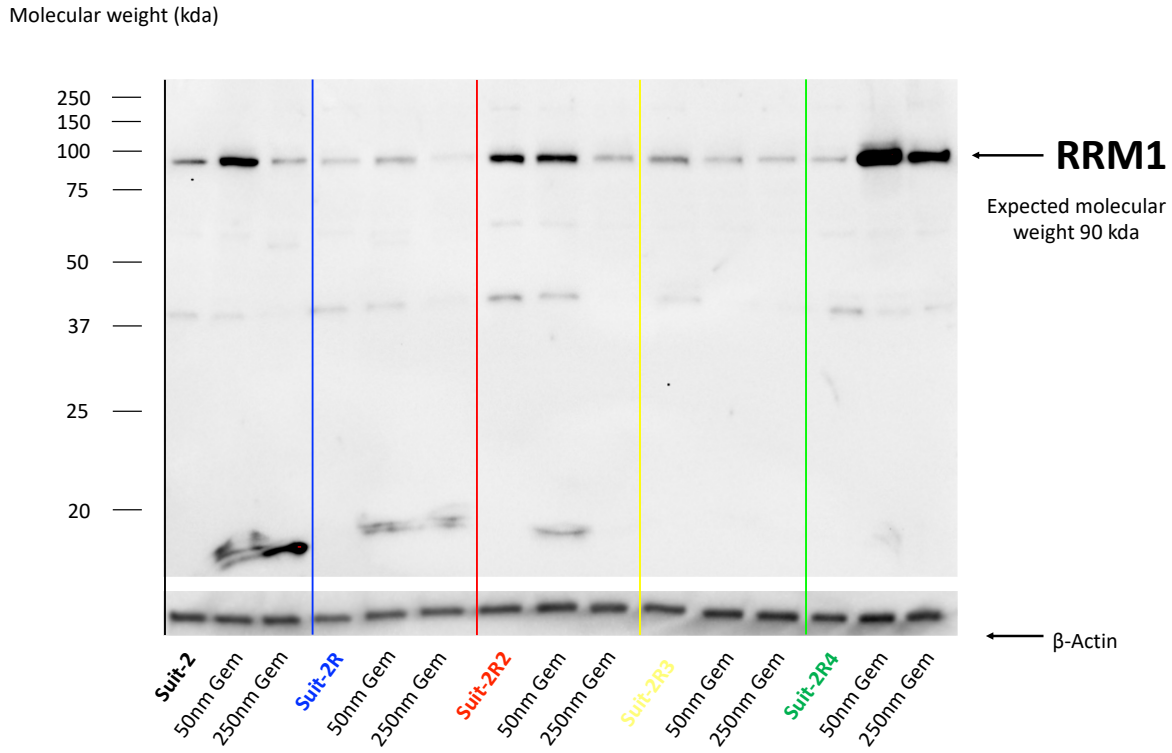
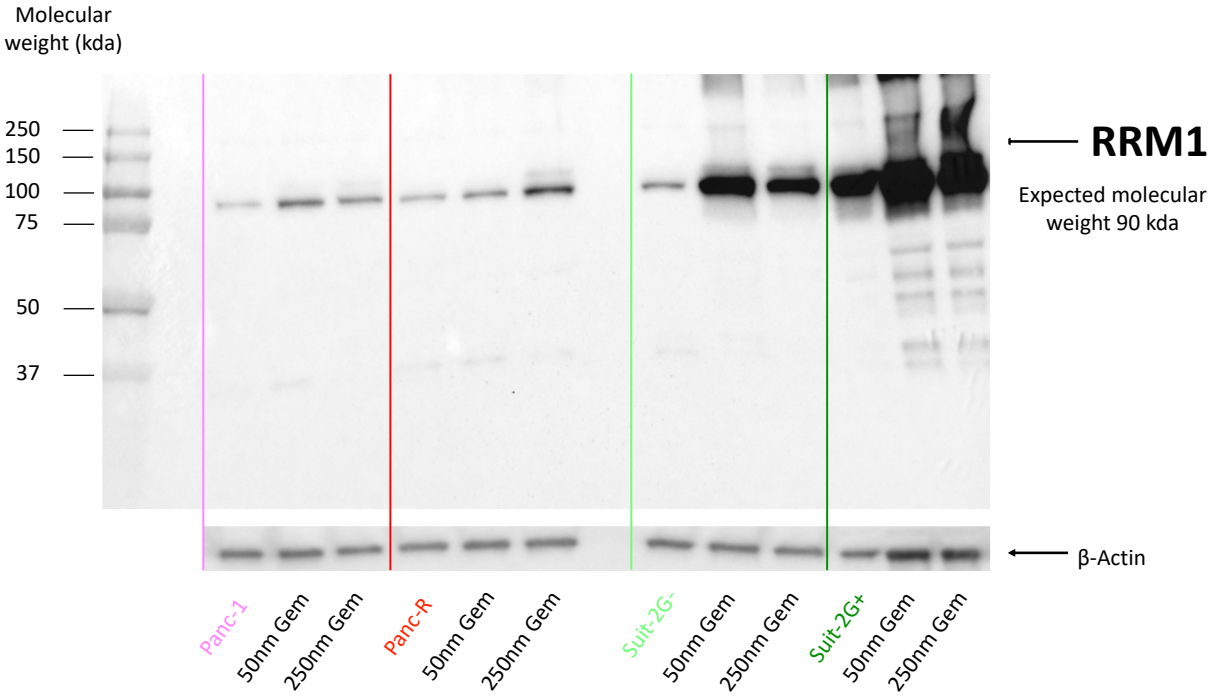


Figure 24 *A western blot to show the expression of the RRM1 protein at the expected molecular weight of 90kDa with treatment parameters of 50nM and 250nM of gemcitabine compared to a control line of a) Suit-2 and clonally resistant lines and b) Suit-2G+, Suit-2G- and Panc-1, Panc-R. the parent cell lines of Suit-2 and Panc-1 demonstrate an increase in RRM1 protein level at the mean IC50 of gemcitabine in resistant lines and then a decrease below that of the control. This effect is seen in Suit-2R and Suit-2R2. The respective increases occur to the untreated control of the particular resistant line as the levels of RRM1 expression in each line are consistent with previous findings. This trend is similar with Suit-2R4, Suit-2G+ and Suit-2G- cells except quite marked increases exist in RRM1 expression and at a higher dose the levels do not drop to that of the untreated control. Suit-2R3 cells exhibit no such increase and there is a reduction in RRM1 expression at both doses of gemcitabine. Panc-R cells exhibit a unique progressive increase with gemcitabine treatment, with the highest expression of RRM1 at 250nM of gemcitabine*

a) Suit-2G+ and Suit-2G-, Panc-1 and Panc-R



In general, the increase production of RRM1 to gemcitabine is expected in order to circumvent the cytostatic binding of gemcitabine to RRM1. In Suit-2R, Suit-2R2 and Suit-2R3 there is a reduction in RRM1 at the higher dose of gemcitabine, presumably because of the general toxic effect of nucleotide starvation rendering the cell incapable of producing RRM1. This is particularly apparent in Suit-2, Suit-2R and Suit-2R2 where the RRM1 expression drops to less than untreated controls at 250nM of gemcitabine. With respect to Suit-2R4, Suit-2G+, Suit-2G- and Panc-1 all depict levels of RRM1 at 250nM of gemcitabine higher than the control. These cell lines are able to maintain higher levels of RRM1 even at higher doses which may represent their higher IC50s compared to Suit-2R and Suit-2R2. An interesting observation is the Suit-2G- cell line which maintains high levels of RRM1 but is no longer resistant to gemcitabine. Reviewing the comet analysis, it could be concluded that the cells are able to repair DSB but unable to overcome the cytostatic effects despite being able to produce high levels of RRM1. Suit-2R2 and Suit-2R4 which both have low levels of RRM1 show little response to gemcitabine treatment suggesting a different form of resistance and relationship to RRM1 compared to other cell lines.

3.5.2 The effect of RRM1 siRNA interference on gemcitabine resistance

Figure 25 demonstrates a successful RRM1 knockdown after 24hrs of RRM1 siRNA treatment of the Suit-2 cell line. This blot also depicts RRM2 and p53R2 knockdown columns which were also analysed through cell viability assays. These knockdowns were successful but not demonstrated here because the MTS assays did show any change and are not be discussed further in this thesis. It should be noted that the additional RRM1 knockdown blots on all resistant cell lines were performed but are not available for this thesis. The MTS assays were of a four-day duration as opposed to three days because of the additional 24hr siRNA treatment required.

There is considerable diversity amongst the cells when analysing the effect of the interference, with a decrease in growth rates except for Suit-2G+. There is a significant reduction in cell growth in Suit-2R2, Suit-2R3, Suit-2G- and to a lesser degree in the remaining lines. This is worth bearing in mind when contemplating figures 27 & 28 and the drug dose curves and when trying to calculate IC50 readings. Figure 26 demonstrates how essential RRM1 is with respect to DNA replication and cellular division.

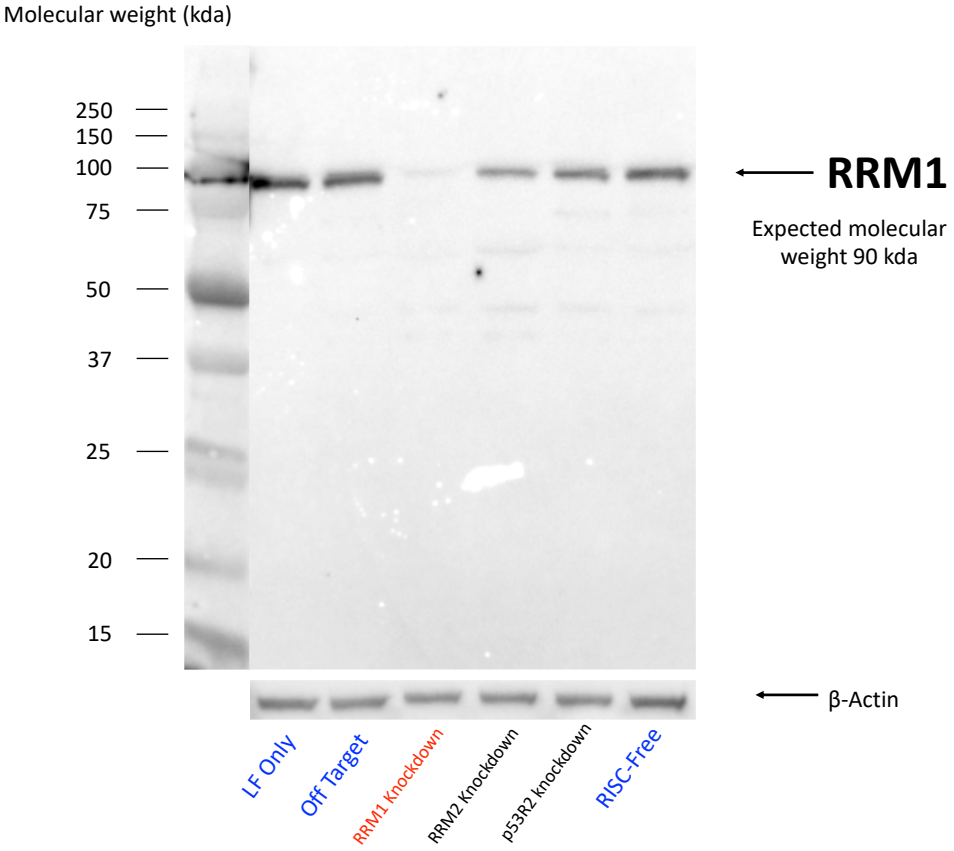


Figure 25 *A western blot to show the expression of the RRM1 protein in Suit-2 cells at the expected molecular weight of 90kDa with treatment parameters of lipofectamine only, Off Target knockdown, RRM1 knockdown, RRM2 knockdown, p53R2 knockdown and RISC-Free knockdown. This blot demonstrates successful knockdown of RRM1 after 24 hours treatment of RRM1 siRNA*

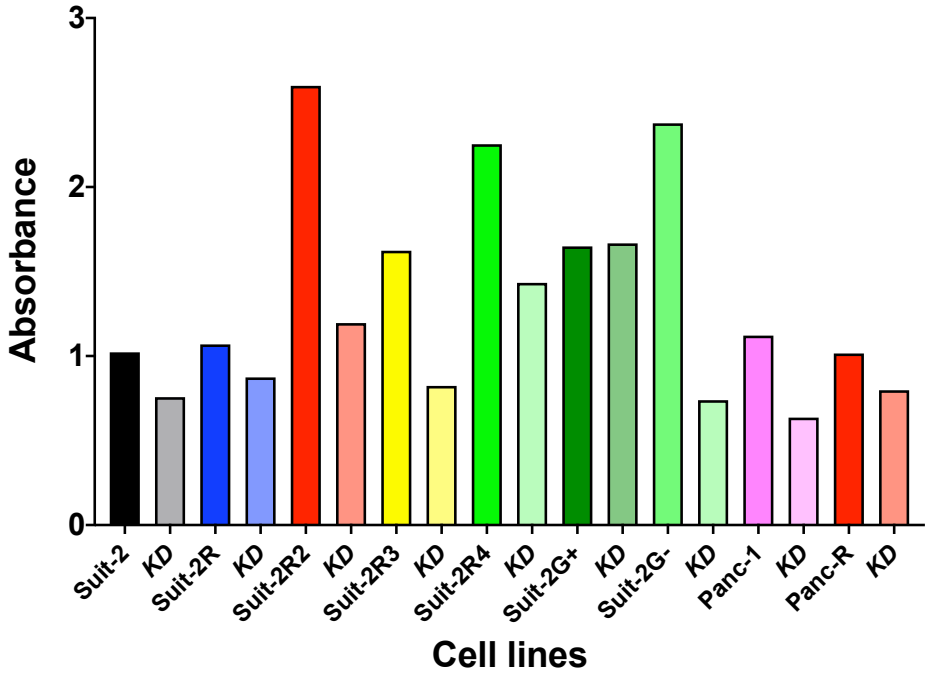


Figure 26 *A bar graph depicting the experimental control arm of the cell viability assay of all cell lines undergoing RRM1 knockdown, as part of the drug response in figures 27 & 28. The absorbance result is a 96-hour snapshot of cell growth and demonstrates the variability of rates between cell lines and the lower rate from this RRM1 knockdown procedure, except for the Suit-2G+ cell line*

Figures 27 and 28 show that RRM1 knockdown has a reduction in sensitivity (curve shifted to left) with respect to the Suit-2 parent cell line with no obvious effect in the Panc-1 parent. All the clonally resistant cell lines including Panc-R do not exhibit any obvious change to resistance as the curves do not deviate from the lipofectamine control, except for Suit-2R4. The curve has clearly shifted to the right with greater vitality at higher doses of gemcitabine, suggesting the resistance in Suit-2R4 is not necessarily solely based on RRM1 overexpression. There is clearly no resistance effect with respect to Suit-2G- but a decrease in growth rate is apparent in figure 26. Suit-2G+ is the only cell line that does not have an effect on viability with RRM1 knockdown and also surprisingly seems to have no impact on resistance. So, despite gemcitabine causing a dramatic increase in RRM1 levels, this does not appear to be the exclusive reason for the resistance. Note that the lipofectamine control has also demonstrated some loss of viability which needs to be taken into consideration when interpreting the data on siRNA. Although the conclusions above are based on relative effect of the RRM1 knockdown against similarly treated cells.

In summary the knockdown of RRM1 has a considerable effect on the growth rate of almost all cell lines which is expected given its role in providing nucleotides to DNA replication, and it also increases sensitivity to gemcitabine in the Suit-2 parent cell line, as would be expected as the target for

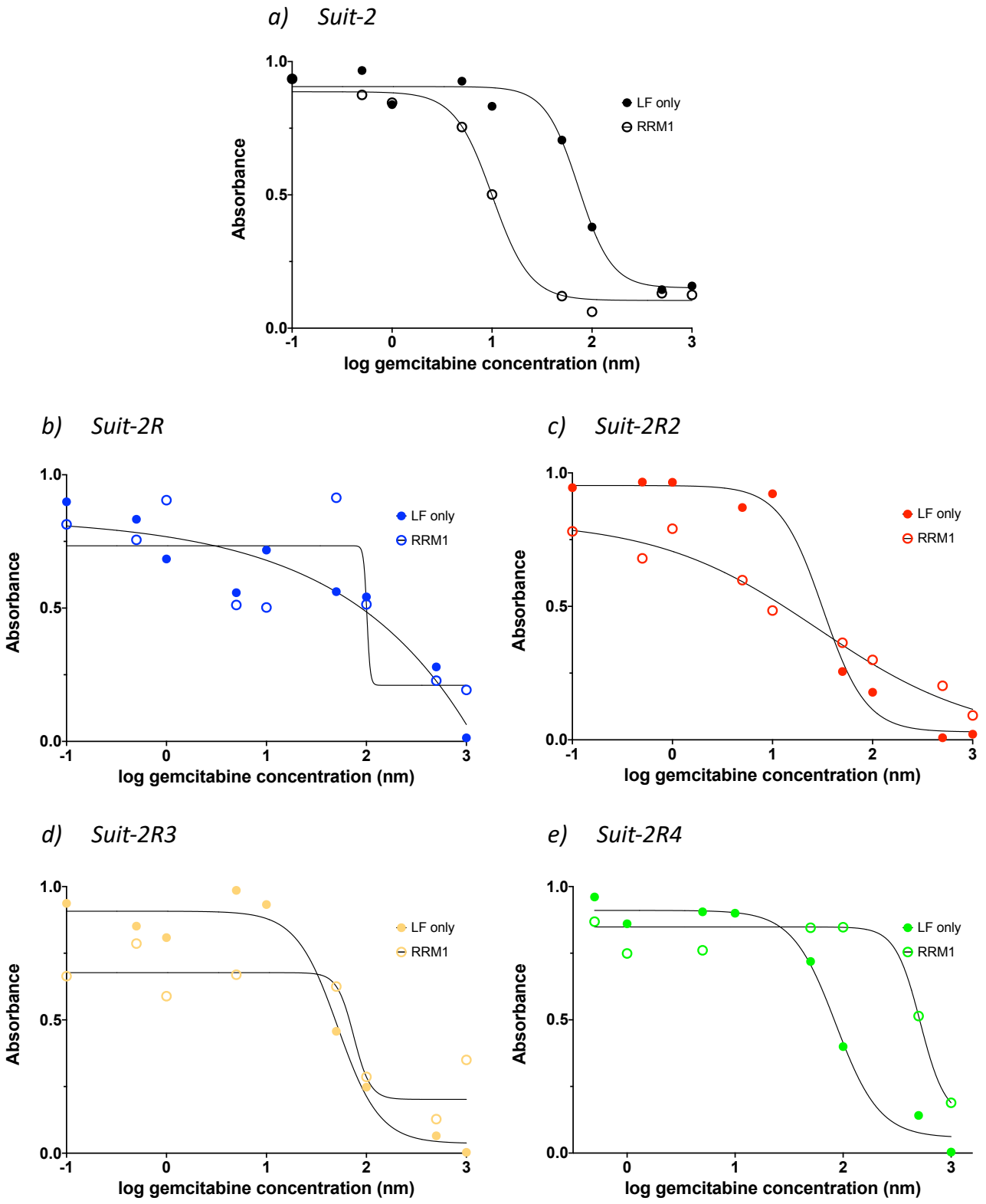


Figure 27 *A cell viability assay of Suit-2 and clonally resistant cell lines Suit-2R, Suit-2R2, Suit-2R3, Suit-2R4 comparing RRM1 knockdown to lipofectamine only control. The absorbance on the y-axis was normalised to a value between zero and one to allow direct comparison of the best fit non-linear regression curve. Figure 26 demonstrates the differences in growth rates of these cell lines which does make drug response curves more difficult to perform. In any case Suit-2 cells exhibit an increase in sensitivity with RRM1 knockdown, an effect not seen in any other cell line. Suit-2R4 potentially exhibits an increase in resistance with the RRM1 knockdown curve to the right*

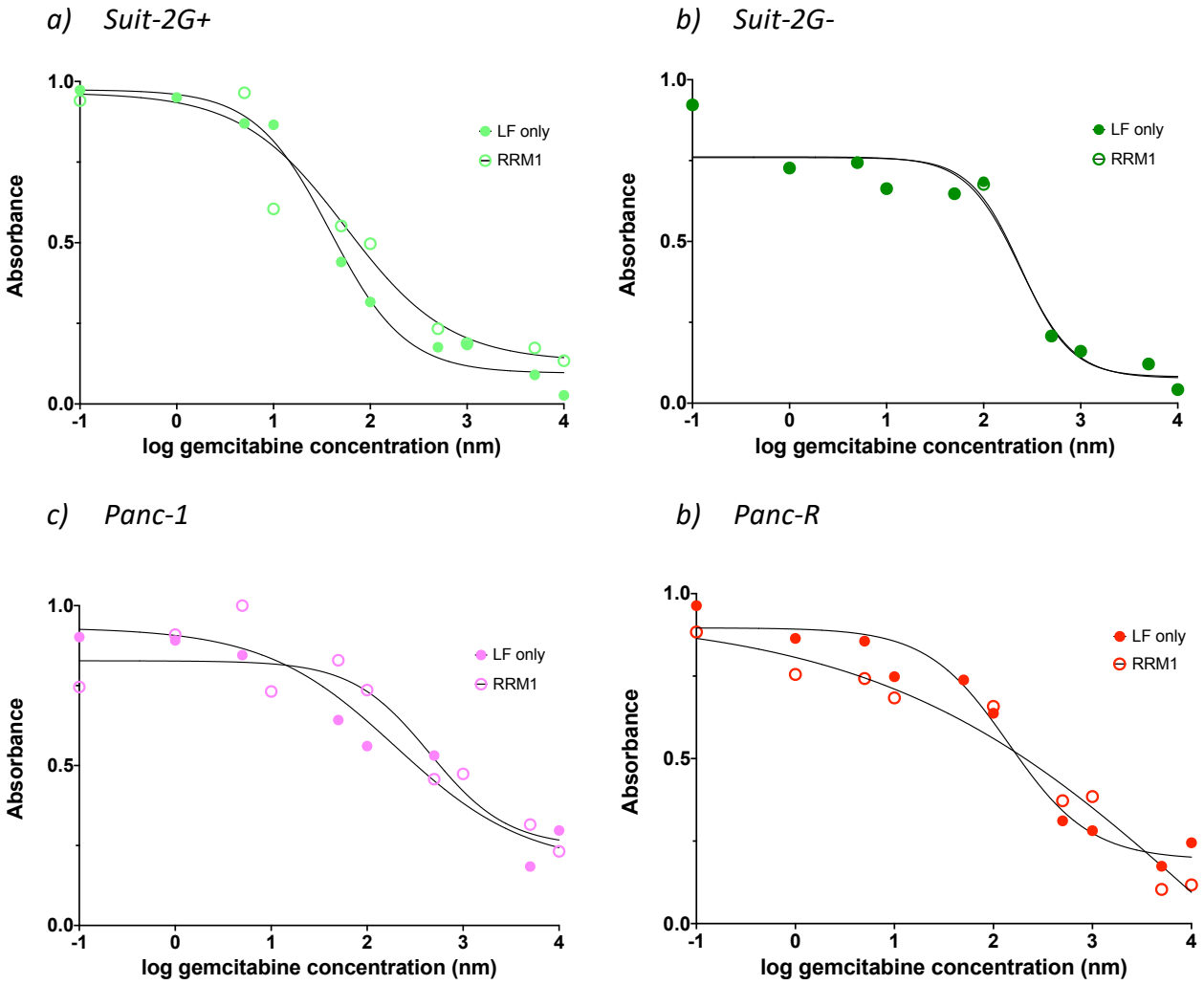


Figure 28 *A cell viability assay of Suit-2G+ and Suit-2G-, Panc-1 and Panc-R comparing RRM1 knockdown to lipofectamine only control. The absorbance on the y-axis was normalised to a value between zero and one to allow direct comparison of the best fit non-linear regression curve. There are no discernible differences in resistance in the Suit-2G lines and knockdown is likely to have not been completely successful or have no effect in Suit-2G+ cells. There are no obvious differences in the dose response curves with the Panc-1 and Panc-R cell lines*

gemcitabine. The resistant cell lines contrast with this in that in no case does the knockdown increase sensitivity, and in Suit-2R4 it is possibly increased.

3.5.3 The effect of RRM1 transfection on gemcitabine resistance

Figure 29 demonstrates the RRM1 transfection effect after 24hrs of RRM1 cDNA treatments compared to the control cell line. The effect of the transfection varied between the cell lines, being most effective in Suit-2R3, Suit-2R4, Panc-1 and least effective in the Suit-2R, Suit-2R2 and Suit-2G cell lines. This may because of different levels of dependency on RRM1 in these cell lines. Figure 30 depicts growth bar charts of each cell lines after the transfection process. There are either no changes observed or small decreases in viability in the cell lines Suit-2, Suit-2R2, Suit-2R3, Suit-2R4, Panc-1 and Panc-R. There are more moderate decreases in vitality seen with the Suit-2R, Suit-2G- and Suit-2G+ cell lines and could be due to the lipofectamine effect described above.

a) Suit-2 and clonally resistant cell lines Suit-2R, Suit-2R2 and Suit-2R3

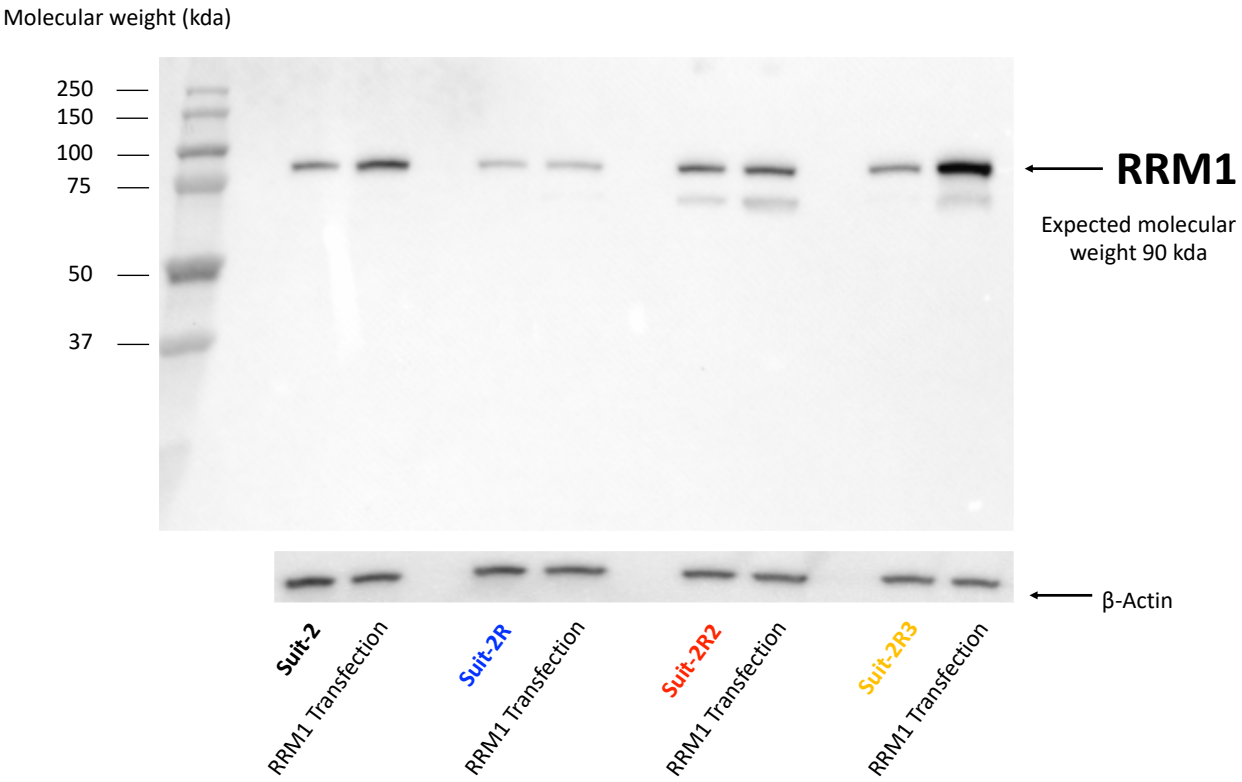


Figure 29 *A western blot to show the expression of the RRM1 protein at the expected molecular weight of 90kDa with treatment parameters RRM1 cDNA transfection compared to control of a) Suit-2, Suit-2R, Suit-2R2, Suit-2R3 and b) Suit-2R4, Suit-2G+, Suit-2G- and Panc-1, Panc-R. There is a general increase in RRM1 protein level in most cell lines demonstrating successful RRM1 RNA transfection. Suit-2R and both Suit-2G lines exhibit no change to RRM1 expression*

b) Suit-2R4, Suit-2G+ and Suit-2G-, Panc-1 and Panc-R

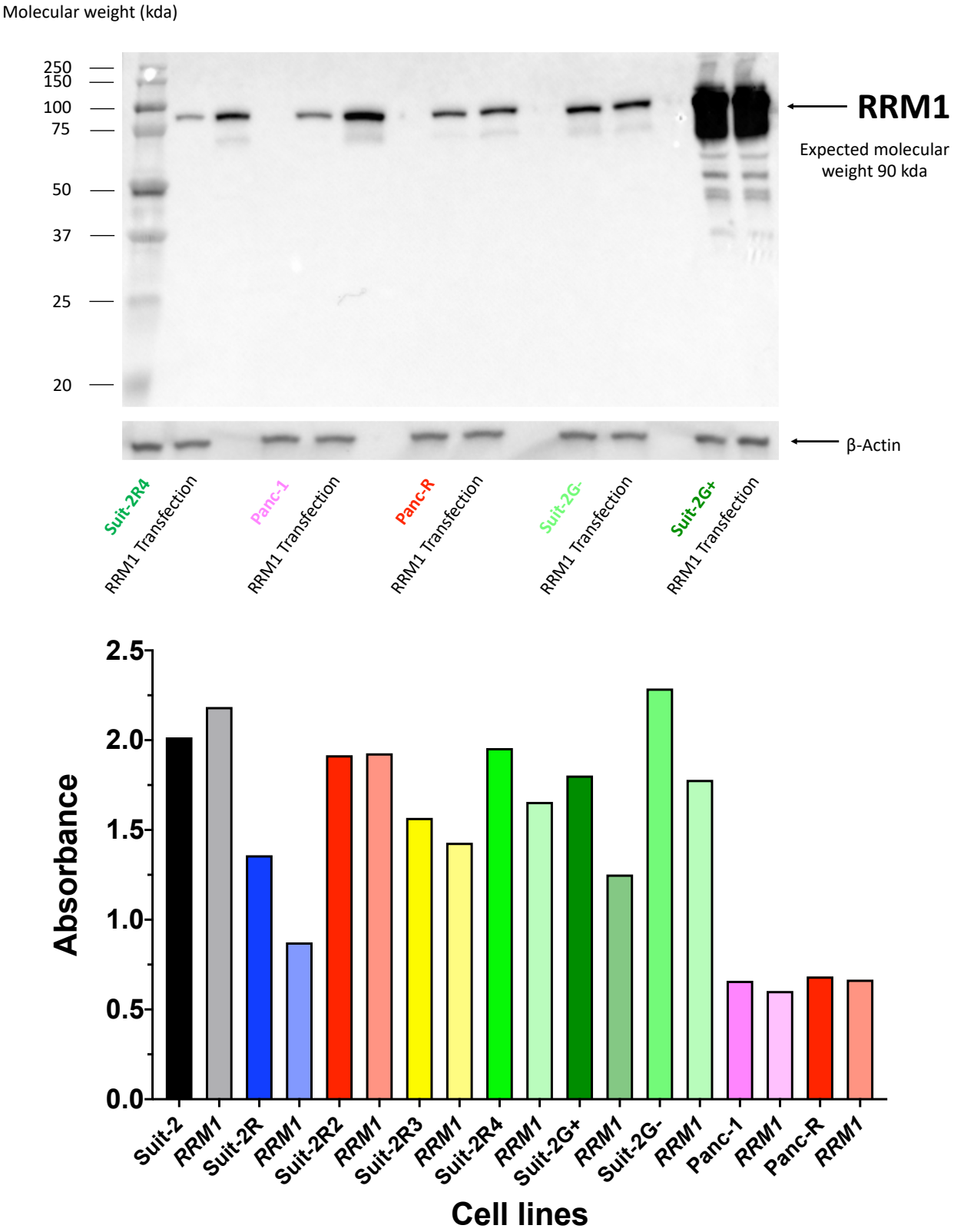


Figure 30 *A bar graph depicting the experimental control arm of the cell viability assay of all cell lines undergoing RRM1 transfection, as part of the drug response in figures 31 & 32. The absorbance result is a 96-hour snapshot of cell growth and demonstrates the variability of rates between cell lines and general stability with RRM1 transfection except with Suit-2R and Suit-2G+ and Suit-2G- cell lines*

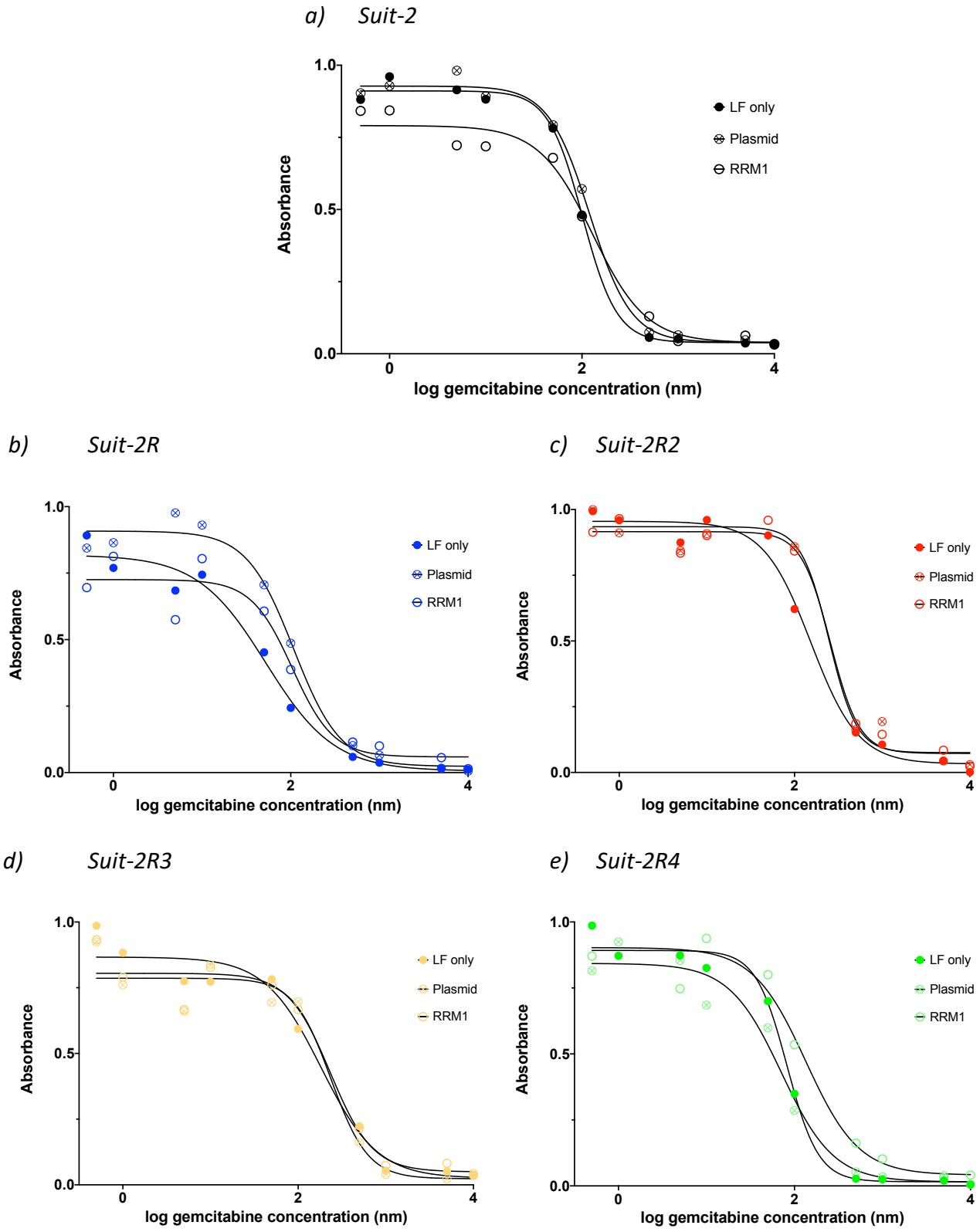


Figure 31 *A cell viability assay of Suit-2 and clonally resistant cell lines Suit-2R, Suit-2R2, Suit-2R3, Suit-2R4 comparing RRM1 transfection to empty plasmid transfection and a lipofectamine only control. The absorbance on the y-axis was normalised to a value between zero and one to allow direct comparison of the best fit non-linear regression curve. There are no discernible differences in resistance in all cell lines though the curve does move to the right with b) Suit-2R c) Suit-2R2 and e) Suit-2R4. In Suit-2R and Suit-2R2 the plasmid control is similarly to the left of the lipofectamine control, negating any significant finding*

Figures 31 and 32 demonstrate the cell viability experiments with drug dose response curves fitted. It is initially worth noting that Suit-2 cells demonstrate no variability in the curve position and subsequently no noticeable change in IC50. This is difficult to assess with regards to Panc-1 as the experiment has not produced clear results, but the RRM1 transfection curve sits between the two control curves, implying no change. Similarly, it is difficult to evaluate the results in Panc-R with no evidence of an alteration in resistance. There is no effect on resistance seen in all the clonal Suit-2 cell lines, except Suit-2R4 which may show a shift to the right compared to the control parameters. Therefore, though knockdown of RRM1, at least in the parent strain (Suit-2), can increase sensitivity to gemcitabine, artificial overexpression makes little difference in any of the cell lines.

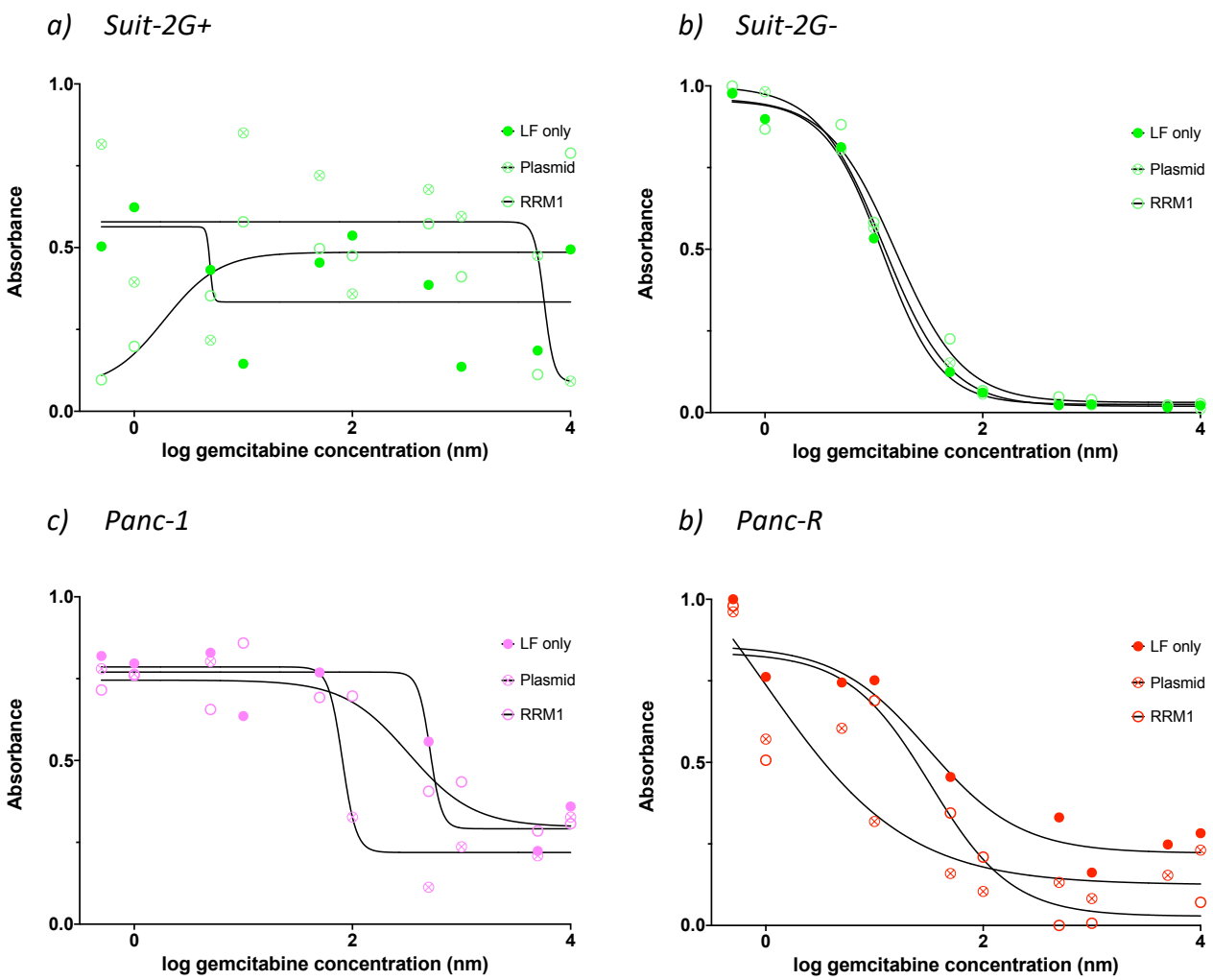


Figure 32 *A cell viability assay of Suit-2G+ and Suit-2G-, Panc-1 and Panc-R RRM1 transfection to empty plasmid transfection and a lipofectamine only control. The absorbance on the y-axis was normalised to a value between zero and one to allow direct comparison of the best fit non-linear regression curve. This was not possible with Suit-2G+ cell as there has been no change to resistance in any of the treatment parameters, hence the lack of a sigmoid curve. The Suit-2G- cell line exhibits a return to Suit-2 with sensitivity to gemcitabine not affected by RRM1 transfection. The Panc-1 and Panc-R cell line do not exhibit clear trends and the transfection process has failed to return a clear assay. It is not possible to ascertain any real effect with the experimental parameters*

In assessing the effect of RRM1 we have determined that there are cellular changes when the cell is exposed to gemcitabine. The experiments above are summarised in Table 7.

Almost all of cell lines respond by increasing the expression of the protein in order to combat both cytotoxic and cytostatic effects of gemcitabine. There is a clear loss of growth when RRM1 is knockdown in cells and no pertinent change when it is transfected, and expression has been shown to increase. Only Suit-2R4 cells show that resistance can be gained through reducing RRM1 expression, the remaining cell lines show no effect outside of growth inhibition. This compares with the increased sensitivity with RRM1 knockdown in the parent Suit-2 cell line. Suit-2R4 and Suit-2G+ are the only cell lines to show the least double stranded DNA damage at high doses of gemcitabine, but noticeable while Suit-2G+ also seemed resistant to gamma radiation, Suit-2R4 seemed to be as sensitive as the parent cell line. Suit-2R4 has a reduced level of RRM1 in its native state whereas Suit-2G+ has a profoundly high level of RRM1 maintained by persistent gemcitabine exposure. There are clearly separate strategies to circumvent the cytostatic effects of gemcitabine and counteract the cytotoxic effects. Suit-2G+ resistance is associated with a large increase in RRM1, although knocking down RRM1 itself did not eliminate resistance. Whilst Suit-2R4 has resistance with a low level of RRM1, perhaps indicating that resistance is not associated with maintaining the nucleotide supply pool. The high RRM1 level concept conferring resistance is consistent in Suit-2R2, Suit-2R3 and Panc-R who have a high expression of RRM1 compared to the parent cell lines. Suit-2R has a slow growth rate, low level of RRM1 and significantly less DNA damage at 250nM of gemcitabine, though it seems equally sensitive to gamma radiation. It is noticeable difference that Suit-2R is a slow growing cell compared to its parent Suit-2 and all other clonally resistant lines. There is likely to be alterations to the cell that affords some to overcome the gemcitabine induced replicative stress. Investigating the cell cycle checkpoint controls may allow further information to be gathered on how cells manage replicative stress and DNA damage to successfully undergo mitosis.

Cell line / Experiment	Degree of resistance	Growth rate	dsDNA damage	RRM1 protein level	Gemcitabine effect on RRM1 expression	RRM1 knockdown cell viability	RRM1 transfection cell viability
Suit-2	-	-	Progressive increase with gemcitabine	-	Increases at 50nM and then decreases at 250nM	Sensitivity to gemcitabine, reduction in growth	Little change in growth rate and no change to sensitivity
Suit-2R	Low	Low	Increases but less than Suit-2	Low expression	Increases at 50nM and then decreases at 250nM	No change to sensitivity, small reduction in growth	Minimal transfection effect Growth rate decreases and no changes to sensitivity
Suit-2R2	Low	High	Increases but less than Suit-2	High expression	Increases at 50nM and then decreases at 250nM	No change to sensitivity, small reduction in growth	Mild decrease to growth rate, no change to sensitivity
Suit-2R3	High	High	Increases but less than Suit-2	High expression	Decreases at 50nM and then further at 250nM	No change to sensitivity, reduction in growth	No change to growth rate or sensitivity
Suit-2R4	High	High	Minimal damage at high doses	Low expression	Increased at 50nM, decreases at 250nM, but still higher than control	Increase in resistance, reduction in growth	No change to growth rate, possibly more resistant
Suit-2G+	Extremely high	High	Minimal damage at high doses	Very high expression	Increases at 50nM and then decreases at 250nM	Develops sensitivity, no change to growth	Minimal transfection effect, growth rate reduced. No change to sensitivity
Suit-2G-	Nil	High	Increases but less than Suit-2 and lower with positive control	High expression	Increases at 50nM and then decreases at 250nM	No change to sensitivity, reduction in growth	Minimal transfection effect & growth rate decreases. No changes to sensitivity
Panc-1	-	Low	Progressive increase with gemcitabine	-	Increases at 50nM and then decreases at 250nM	No change to sensitivity, reduction in growth	No change to growth or sensitivity
Panc-R	High	Low	Increases but less than Suit-2	High expression	Increases at 50nM and at 250nM	No change to sensitivity, small reduction in growth	Minimal transfection effect & no change to growth or sensitivity

Table 7 Summarising the experiments outcomes investigating the cytotoxic and cytostatic effects of gemcitabine on all resistant cell lines

3.6 The role of cell cycle checkpoint markers in the resistance of gemcitabine

There are multiple checkpoint markers that have been implicated in pancreatic tumourigenesis and the key markers chosen for analysis are those integral to regulating DNA replication and cell cycle progression. Cell cycle proteins were selected for investigation based upon literature review and published relationships either with gemcitabine or with ribonucleotide reductase, as discussed in the introduction (77-84).

3.6.1 The role of p21 in resistant cell lines

Figure 33 demonstrates the mean fold change in p21 RNA production. There is a marked fold reduction in all Suit-2R clonally resistant cells compared to the parent Suit-2 cell line: Suit-2R 0.49, Suit-2R2 0.50, Suit-2R3 0.41, Suit-2R4 0.37-fold change. There are no changes in expression to the Suit-2G+ cell line compared to Suit-2 and there is a 1.36-fold increase in p21 RNA in Panc-R cells compared to Panc-1. The effect on protein level, of the widespread pertinent decrease in p21 RNA in the Suit-2 clonally resistant cell lines, is reviewed in figure 34. All but Suit-2R3 exhibit lower level of p21 protein level compared to Suit-2, and the Suit-R3 line demonstrates a similar level of protein. The Suit-2G cell lines also exhibit a lower p21 protein level intimating an epigenetic alteration. The Panc-R line has similar findings at a protein level that were seen at an RNA level (figure 34).

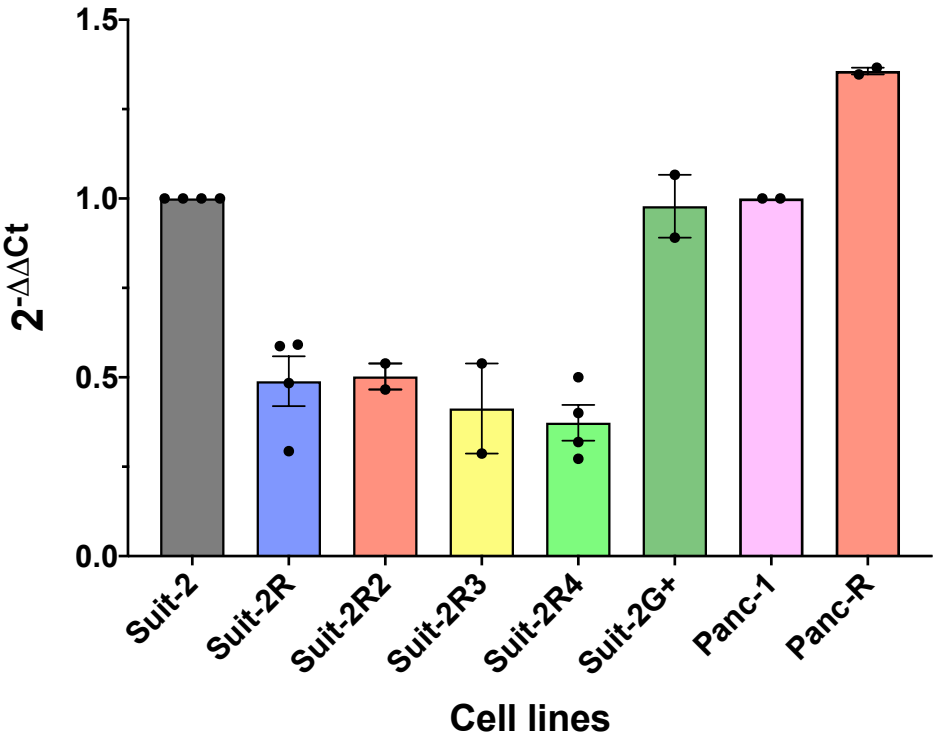


Figure 33 A *bar chart to demonstrate the mean fold change of p21 RNA expression in Suit-2 and Panc-1 with the clonally and adaptively resistant cell lines. Each dot represents an experimental replicate and mean and standard errors are plotted for each cell lines. Suit-2R, Suit-2R2, Suit-2R3, Suit-2R4 all demonstrate at least a two-fold reduction in p21 RNA compared to Suit-2. This contrasts with Suit-2G+ which shows no changes and Panc-R cells exhibit an increase in p21 RNA expression.*

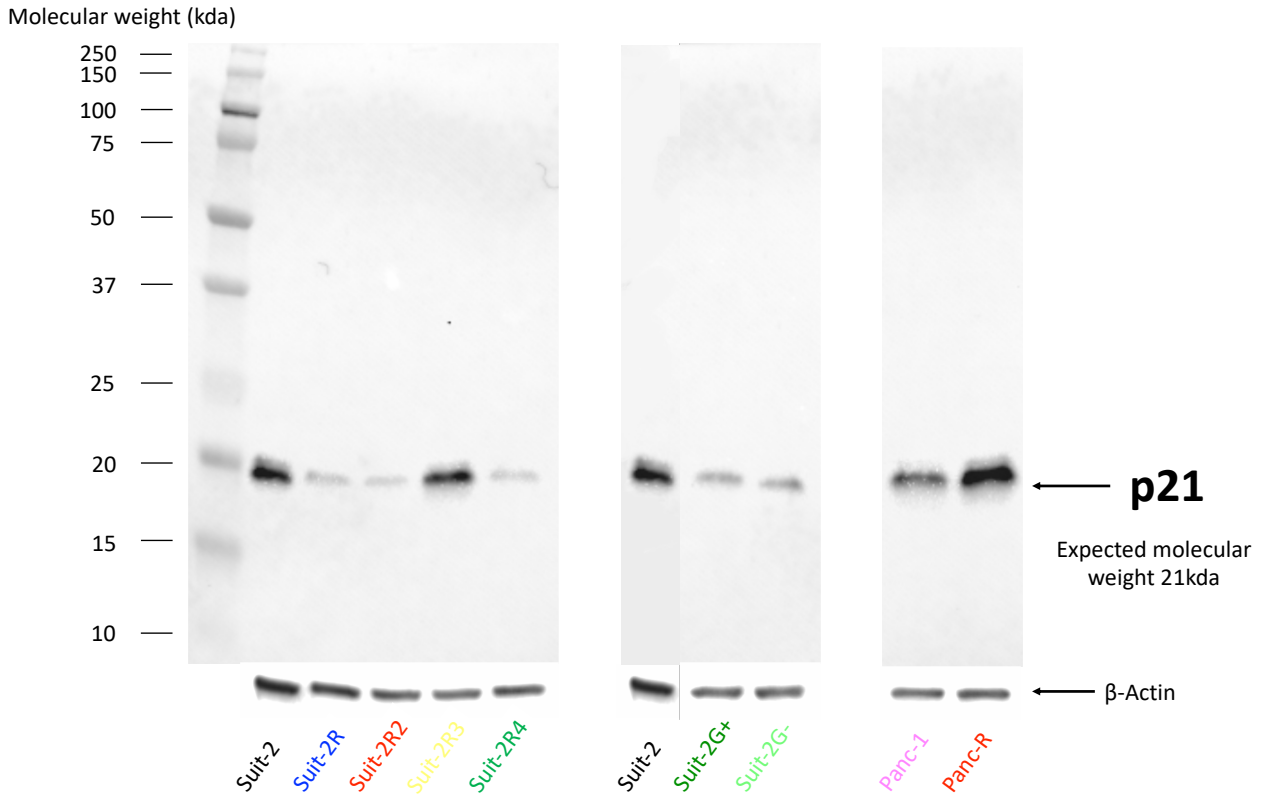


Figure 34 *A western blot to show the expression of the p21 protein at the expected molecular weight of 21kDa, with the housekeeping protein β-actin at the base of the blot. The Suit-2R lines show reduced protein level, except Suit-2R3 which is similar to Suit-2. Suit-2G+ and Suit-2G- both show a reduced expression. Panc-R does exhibit a clear increase in expression*

p21 has a multiple cell cycle functions and increases with DNA damage to halt the G1/S transition but also serves as part of an anti-apoptotic cascade. The role of p21 is to halt the cell cycle and the reduced expression may indicate a drive to prevent cell cycle inhibition. However, if cells are unable to undergo mitosis because of replicative stress and a reduction in the nucleotide pool, there will naturally lower levels of p21. The resistance mechanisms in the clonally resistant cells may either be independent of p21 induction with rigid cell cycle control (Suit-2R, Suit-2R2, Suit-2R4, Suit-2G) or p21 may be important in protecting cells from apoptosis and progressing them through the cell cycle albeit it inappropriately. There is a large regulatory system and p21 plays a part of those processes and these cell lines utilise these in different ways. Both halting the G1/S transition and apoptosis are consequences of DNA damage and part of the mechanism to overcome the effects of gemcitabine may involve the p21 related checkpoint markers.

3.6.2 The role of Chk1 in resistant cell lines

Figure 35 demonstrates the mean fold change in RNA expression of Chk1 in resistant cell lines. Suit-2R, Suit-2R2 and Suit-2R3 have lower levels of Chk1 RNA at 0.72, 0.45 and 0.71-fold decreases but Suit-2R4 shows a two-fold increase in expression in comparison to Suit-2. Suit-2G+ cells exhibit no changes compared to the parent line. Panc-R has a two-fold reduction in Chk1 RNA expression. With respect to the impact on protein levels, figure 36 shows potential increases in Chk1 protein level in the Suit-2R2, Suit-2R4, Panc-R and most markedly in the Suit-2R3 cell lines. Suit-2R4 exhibits increased RNA expression but this does not translate to protein levels implying there is a post-translational modification to prevent the cell from arresting. The actin levels of Suit-2 are more marked compared to the other cell lines, which may lead to the conclusion that the Suit-2 G- and Suit-2G+ show no changes in expression, similar to the RNA fold changes. There certainly a reduced expression of Chk1 in the Suit-2R line.

Chk1 has a role at every cell phase and mediates DNA repair mechanisms. The DDR pathway is activated by the ATM and ATR proteins and given the likely gemcitabine induced DSB, the Chk1 marker is likely to be integral to affording time for repair by way of cell cycle arrest or by preventing arrest and accepting a degree of damage in order to pursue cell growth. The variations seen in checkpoint markers so far imply different mechanisms to resistance. The Suit-2G line demonstrate no change implying continued cell growth whereas as Suit-2R4 has dampened down the genetic response in order to prevent arrest. Suit-2R has low level Chk1 and slow growth which may relate to a generalised cell cycle slow down, not specifically to Chk1 phase progression. These findings have to be combined with other markers and reviewed specifically to each resistant cell line, as is demonstrated onwards.

Figure 35 A *bar chart to demonstrate the mean fold change of Chk1 RNA expression in Suit-2 and Panc-1 with the clonally and adaptively resistant cell lines. Each dot represents an experimental replicate and mean and standard errors are plotted for each cell lines. The clonally resistant cell lines, Suit-2R, Suit-2R2, Suit-2R3 and Panc-R all exhibit reductions in Chk1 RNA expression. There are no obvious changes in the Suit-2G+ lines and Suit-2R4 is the only cell line that show an increase in RNA expression compared to the parent cell line*

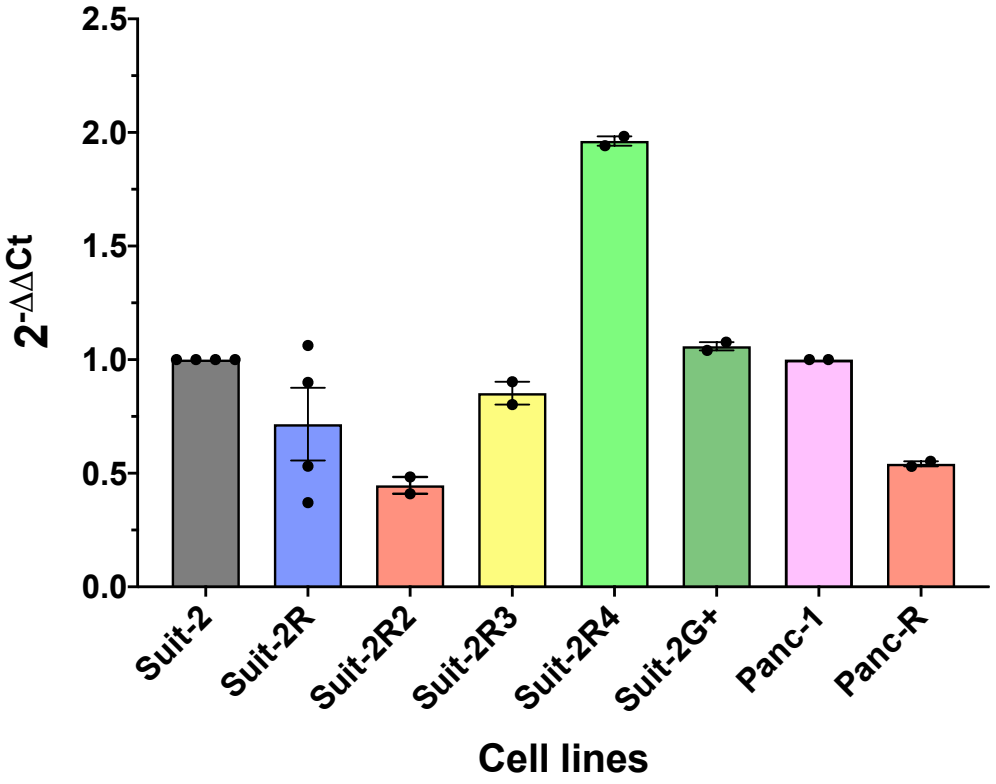
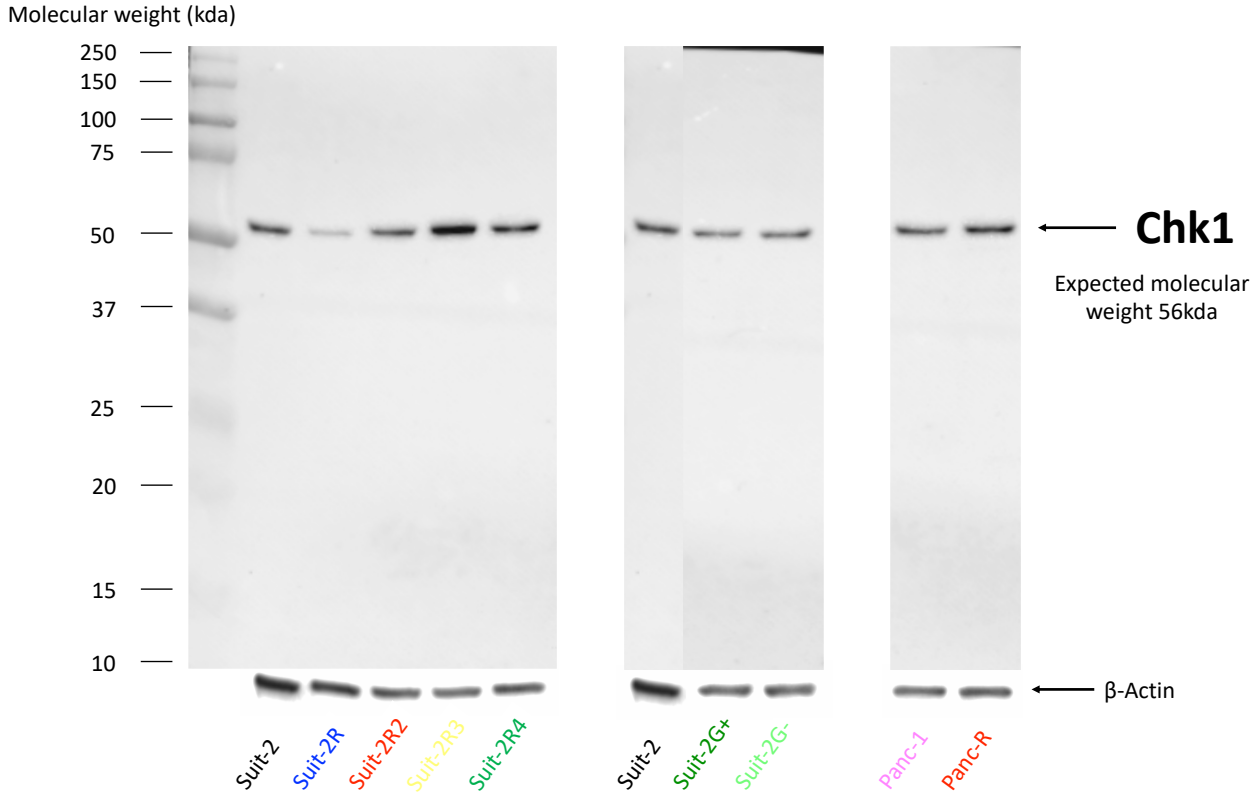


Figure 36 *A western blot to show the expression of the Chk1 protein at the expected molecular weight of 56kDa, with the housekeeping protein β-actin at the base of the blot. There is a higher level of actin expression in the Suit-2 line which does affect the interpretation of the blot, but it is clear that Suit-2R3 and Panc-R are higher in protein levels. This also may lead to the conclusion that Suit-2R2 and Suit-2R4 have increased expression or a the very least similar levels to the parent Suit-2 line. The Suit-2G lines have similar expressions and is unlikely to be different to the Suit-2 line*



3.6.3 The role of CyclinD1 in resistant cell lines

The presence of the G870A polymorphism will have an effect on the protein levels of Cyclin D1a and D1b and the presence of the minor and major allele were investigated by SNP genotyping shown in Figure 37. Part B demonstrates the immortalised cell lines Suit-2, Panc-1 and BxPC3 with homozygous minor, heterozygous and homozygous major alleles respectively. Part A confirms these findings with the Suit-2, Suit-2R, Suit-2R2, Panc-1 and Panc-R cell lines used in this thesis. Cyclin D1a and D1b were investigated separately to elicit the predominance of the variant in the homozygous minor allele of Suit-2. Cyclin D1 RNA analysis was not carried out for the Panc-1 cell line.

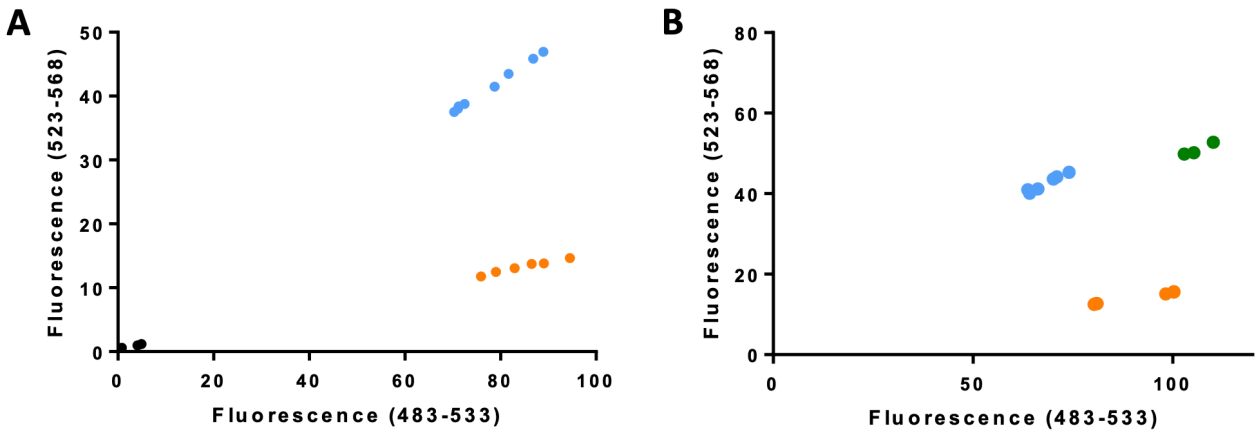


Figure 37 ***A:*** *SNP genotyping of G870 polymorphism in Suit-2, Suit-2R and Suit-2R2 cells (all are blue dots (triplicate), homozygous minor allele), Panc-1 and Panc-R (all orange dots (triplicate), homozygous major allele) with sterile water controls (black dots (triplicate).* ***B:*** *SNP genotyping of Suit-2 (blue (triplicate, two different passages of Suit-2), homozygous minor allele), BxPC3 (green dots (triplicate), heterozygous) and Panc-1 (orange dots (triplicate), homozygous major allele).*

Figure 38 demonstrates the mean fold change in RNA expression of Cyclin D1a in the Suit-2 cell lines. There is a cell line wide increase in mean RNA fold change: Suit-2R 5.13, Suit-2R2 7.95, Suit-2R3 1.83 and Suit-2R4 1.479. It would be pertinent to compare this to the Cyclin D1b RNA expression as seen in figure 39. There are similar patterns in that Suit-2R and Suit-2R2 both exhibit significantly larger mean fold change increases of 17.02 and 17.50 respectively. Suit-2R3 and Suit-2R4 exhibit a smaller increase in expression compared to the parent; 7.37- and 6.41-fold changes respectively. From an RNA expression perspective there are increases in Cyclin D1a and D1b, predominately in the spliced variant form highlighting its importance to the cell cycle. Figure 40 investigates the protein levels of Cyclin D1a and Cyclin D1b in all resistant cell lines and is the same blot at two exposures to enable optimal comparison of both bands.

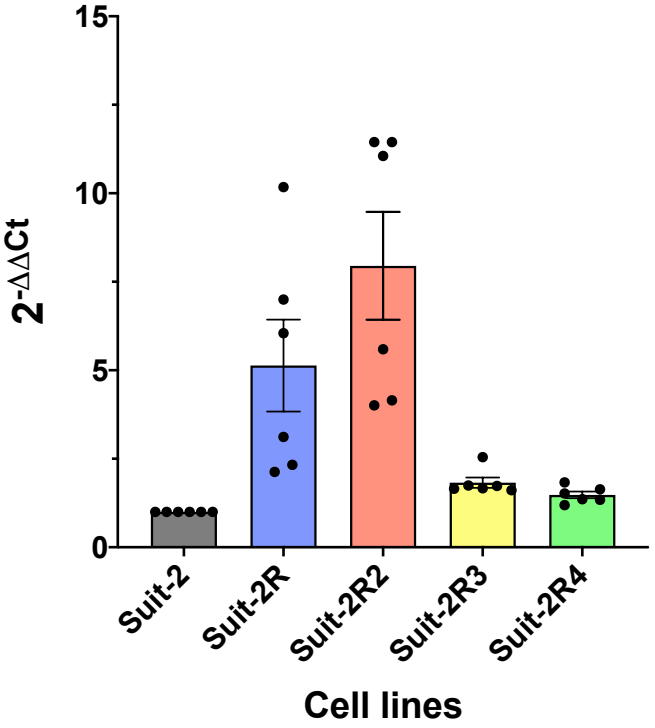


Figure 38 *Bar charts to demonstrate the mean fold change of CCND1a RNA expression in a) Suit-2 and the clonally resistant cell lines. Each dot represents an experimental replicate and mean and standard errors are plotted for each cell lines. Though there is widescale increase in mean RNA expression, there are large increases with respect to Suit-2R and Suit-2R2.*

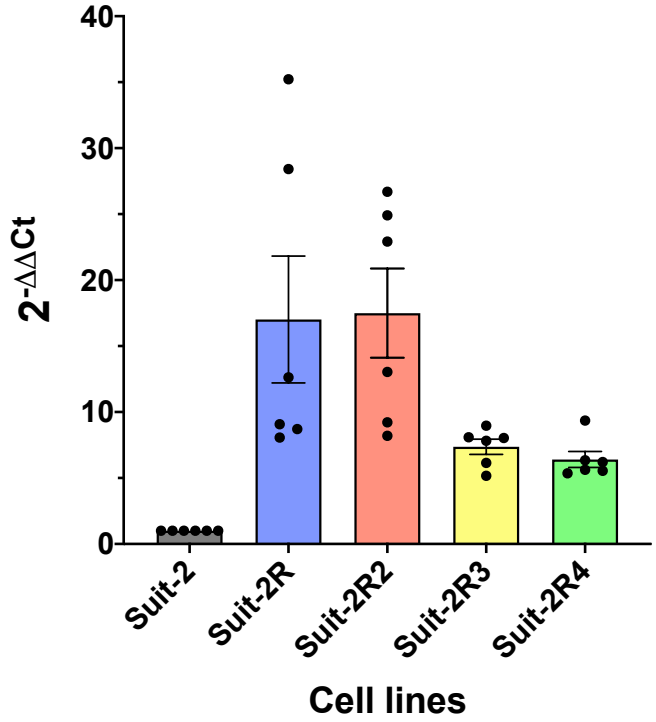
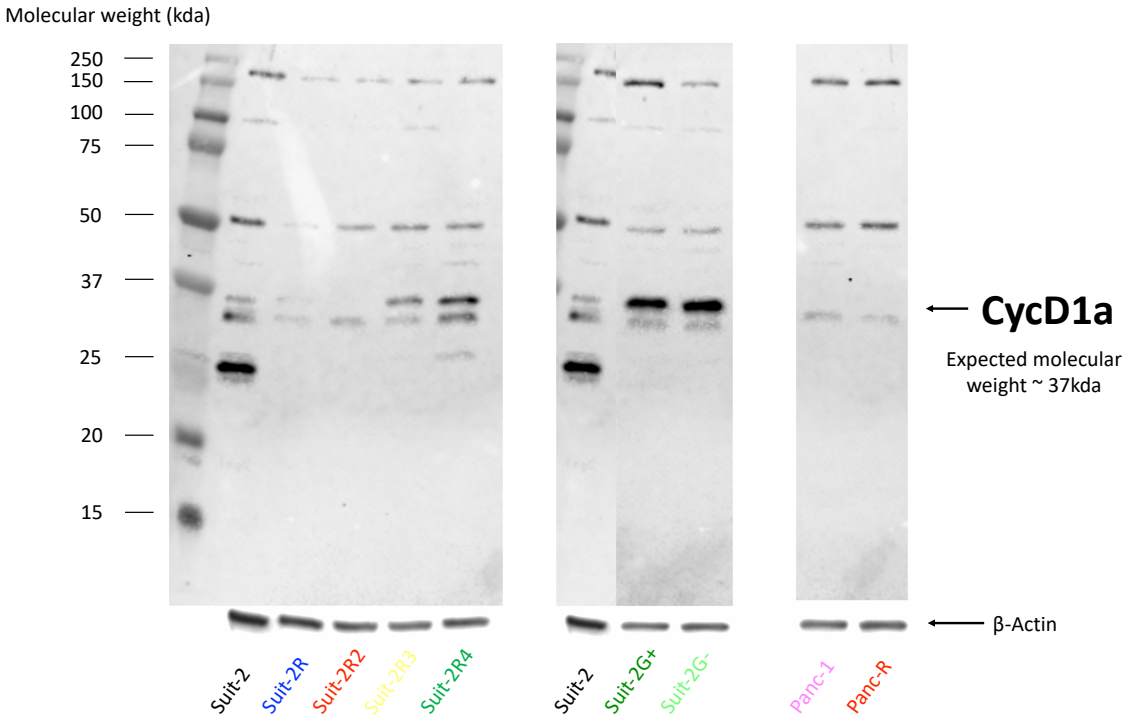


Figure 39 *Bar charts to demonstrate the mean fold change of CCND1b RNA expression in a) Suit-2 and the clonally resistant cell lines. Each dot represents an experimental replicate and mean and standard errors are plotted for each cell lines. There are significant increases in RNA expression across all lines. Similar to figure 33, there are widescale increases in mean RNA expression, largest in the Suit-2R and Suit-2R2 cell lines.*

a)



b)

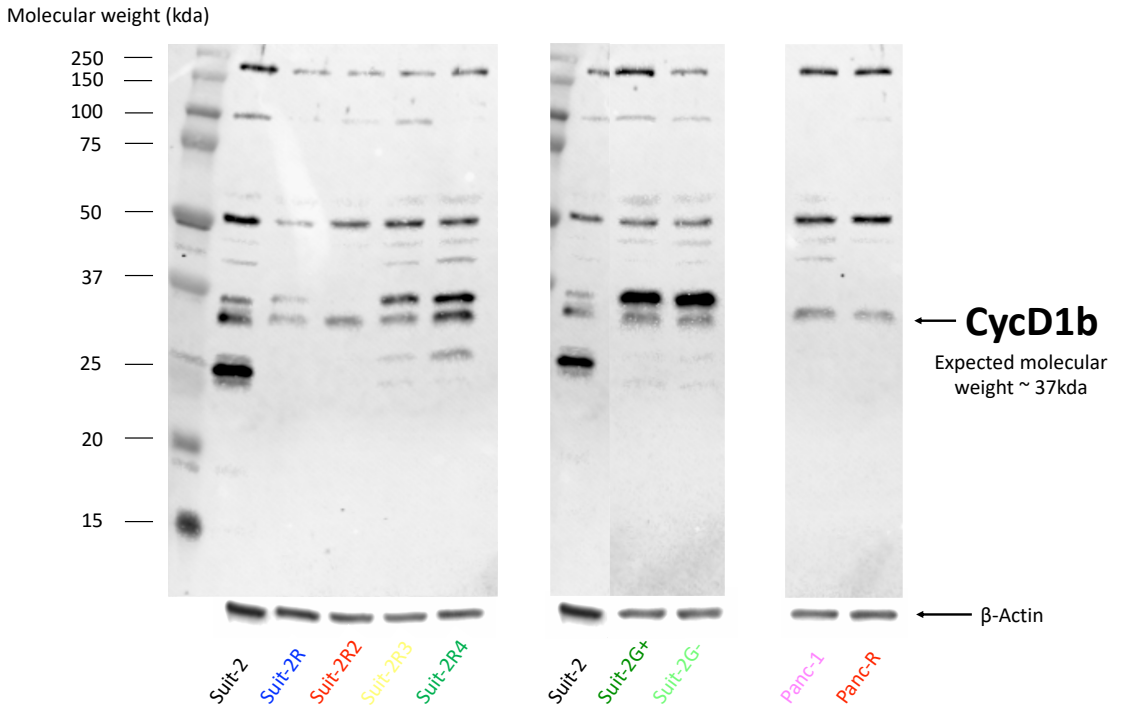


Figure 40a&b *A single western blot at two exposures to contrast the expression of the Cyclin D1a and D1b at the expected molecular weights of 37kDa with D1b being lighter, with the housekeeping protein β-actin at the base of the blot. The Suit-2R and Suit-2R2 lines exhibits decreases in both variants, more so in D1a, whereas Suit-2R3 and Suit-2R4 both have increased expression, more so in D1a. Both Suit-G lines show a large increase in Cyclin D1a and smaller increases in Cyclin D1b. Panc-1 cell lines exhibit only the D1b splice variant with a possible lower level with the Panc-R line*

There are two trends that exist in the Suit-2 clonally resistant cell lines which correlate with the RNA expression. Suit-2R and Suit-2R2 both exhibit increases in D1a and D1b RNA expression which does not translate to increased protein levels. Indeed, both D1a and D1b are reduced compared to the parent Suit-2 line. Cyclin D1b had more prominent protein levels compared to D1a, in line with the higher RNA fold change in this spliced variant. The second trend exists with Suit-2R3 and Suit-2R4 where there is less substantial increase in RNA expression, with Cyclin D1b demonstrating a higher fold change. In these cell lines, the Cyclin D1a D1b protein levels are higher compared to the parent. Despite the fact that the D1b RNA expression being higher it is the protein level of D1a that shows the greatest increase. The Suit-2G+ and Suit-2G- cell lines both exhibit a significant increase in Cyclin D1a compared to Suit-2 cells with no obvious difference with D1b.

There is a clear different in Cyclin D1 in these cell lines, and the impact of the splice variant is likely to play an important role with the clonally resistant line, especially considering its relative redundancy compared to D1a in the Suit-2G cell lines.

Panc-1 cells produce no Cyclin D1a and only express D1b which is related to its major allele G polymorphism. There is little difference with respect to the Panc-R Cyclin D1b expression suggesting that Cyclin D1 may not play a prominent role in the resistance to gemcitabine in this cell line.
Given the marked findings in Cyclin D1a and D1b expression, there is likely to be an important role in the mechanism of gemcitabine which may be related to growth rates and cell cycle regulation in Suit-2. This is probably related to the homozygous minor allele in this cell line.

3.6.4 The effect of Cyclin D1 siRNA interference on gemcitabine resistance

The accumulation of Cyclin D1 is essential for transition into S phase and knocking down this protein should induce cell cycle arrest and may affect how resistant cells are to gemcitabine. Figure 41 shows a western blot of a Cyclin D1 knockdown compared to controls and figure 42 demonstrates a 96hr snapshot into cell growth. It depicts that Suit-2R has almost no change in growth rates compared to the parent Suit-2 cell line that has a 59% drop compared to control. Cell growth drops by 50% in the Suit-2R2, Suit-2R4 and Suit-2G cell lines and by up to 25% in Suit-2R3, Panc-1 and Panc-R cell lines. Though it is difficult to ascertain the exact cause of this drop, the fact that Suit-2 sees no change may allow the conclusion it is related to the lack of Cyclin D1 and not just from the lipofectamine experimental conditions of the control.

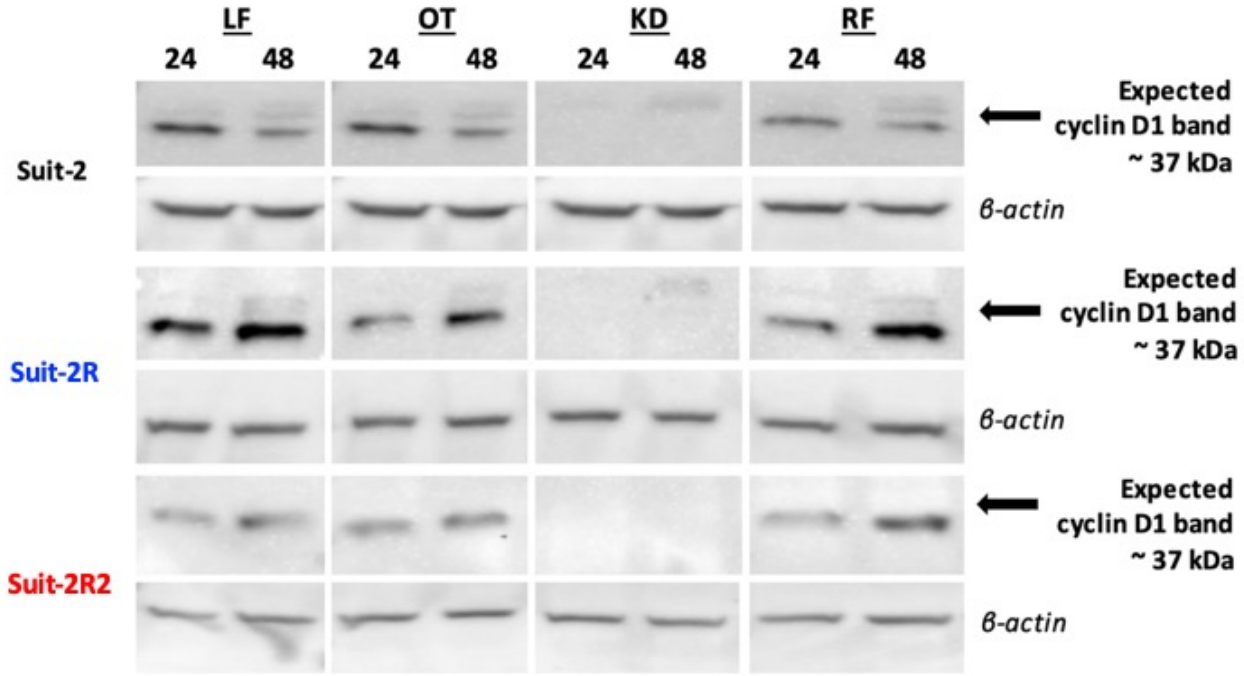


Figure 41 *A western blot to show the expression of the cyclin D1 protein in Suit-2 cells at the expected molecular weight of 37kDa with treatment parameters of lipofectamine only (LF), Off Target knockdown (OT) and RISC-Free (RF) knockdown. This blot demonstrates successful knockdown of cyclin D1 after 24 and 48 hours treatment of cyclin D1 siRNA, in Suit-2, Suit-2R and Suit-2R2 cell lines*

Figures 43 and 44 depict cell viability curves for the Cyclin D1 interference assays and it is worth commenting from the outset that actual dose response curves proved difficult in many cases due to the detrimental effects of the knockdown. With the parent strain, knockdown of Cyclin D1 results in an increase in sensitivity to gemcitabine at lower doses. Whereas there is no impact to Suit-2R, and in Suit-2R2 and Suit-2R3 may even increase resistance. Only in Suit-2R4 is there a possibility of increased sensitivity. From figure 42, it can be seen that the slowing of growth is not additive or synergistic with the impact of gemcitabine, suggesting that the resistance mechanism in Suit-2R and possibly Suit-2R2 and Suit-2R3 are associated with a resistance to cell growth inhibition by gemcitabine, and may even benefit. This contrasts with Suit-2R4 where the inhibition of cell growth does not seem to protect from gemcitabine, and may enhance its effect. Panc-1 and Panc-R cell lines do demonstrate some general reduction in growth with the impact on gemcitabine resistance is difficult to evaluate as there doesn't seem to be a difference between resistant and parent cell line. The Suit-2G cell lines show that the knockdown and lipofectamine treatment required prolonged culture time and there is clearly a transition from G+ to G-. This experiment shows that the interference process brings a sensitivity to gemcitabine independent of the knockdown. This effect is also seen in figure 28.

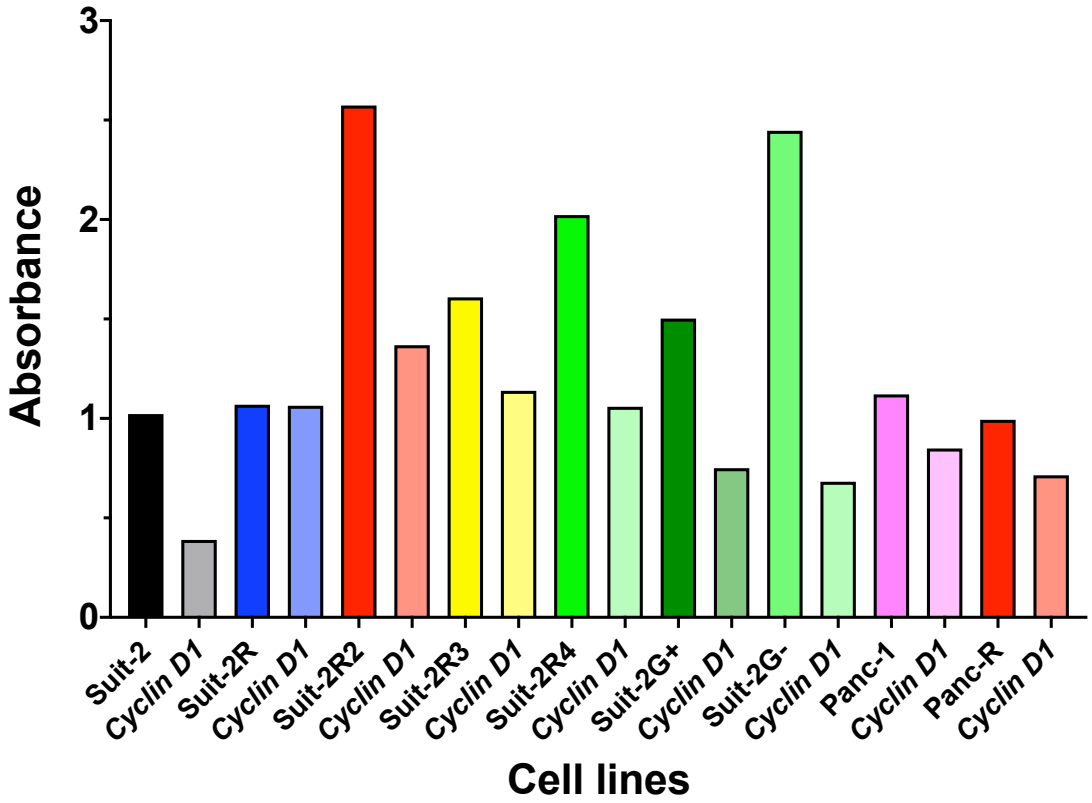


Figure 42 *A bar graph depicting the experimental control arm of the cell viability assay of all cell lines undergoing RRM1 transfection, as part of the drug response in figures 37 & 38. The absorbance result is a 96-hour snapshot of cell growth and demonstrates the general decrease in rates across all cell lines except Suit-2R. There are lesser reductions in growth with respect to Suit-2R3, Panc-1 and Panc-R but even the more significantly affected cell lines are less that the 59% reduction seen in the parent Suit-2 cell line.*

Half of these curves fail to produce satisfactory drug responses and conclusions are difficult to elicit. Some cell lines with low Cyclin D1 demonstrate no effects to growth or potentially some changes to resistance. The role of Cyclin D1 cell cycle regulation to gemcitabine resistance is not clear.

The three cell cycle regulatory markers analysed here are part of a complex cascade that results in either progression through the cell cycle, an arrest to afford repair mechanisms or driving apoptosis of the cell. There appears to be in this respect at least two resistance patterns exemplified by Suit-2R and Suit-2R4, with the former dependent upon or at least not adversely affected by cell cycle inhibition. The contrasting mechanism of Suit-2R4 is antagonised by cell cycle inhibition and is not augmented by this process. The genotype (derived from the parent cell line) would promote the expression of cyclin D1b. Table 8 summarises the checkpoint marker observations in all resistant cell lines. In Suit-2R. In Suit-2R the cyclin D1b expression is evident but the total level of cyclin D1 is reduced, potentially as part of the resistance mechanism. In contrast in Suit-2R4 the preference for D1b is lost despite the cell line continuing to have the minor allele, and at the RNA level the cell line produces more of this spliced variant. Post-transcriptionally that variant downregulated.

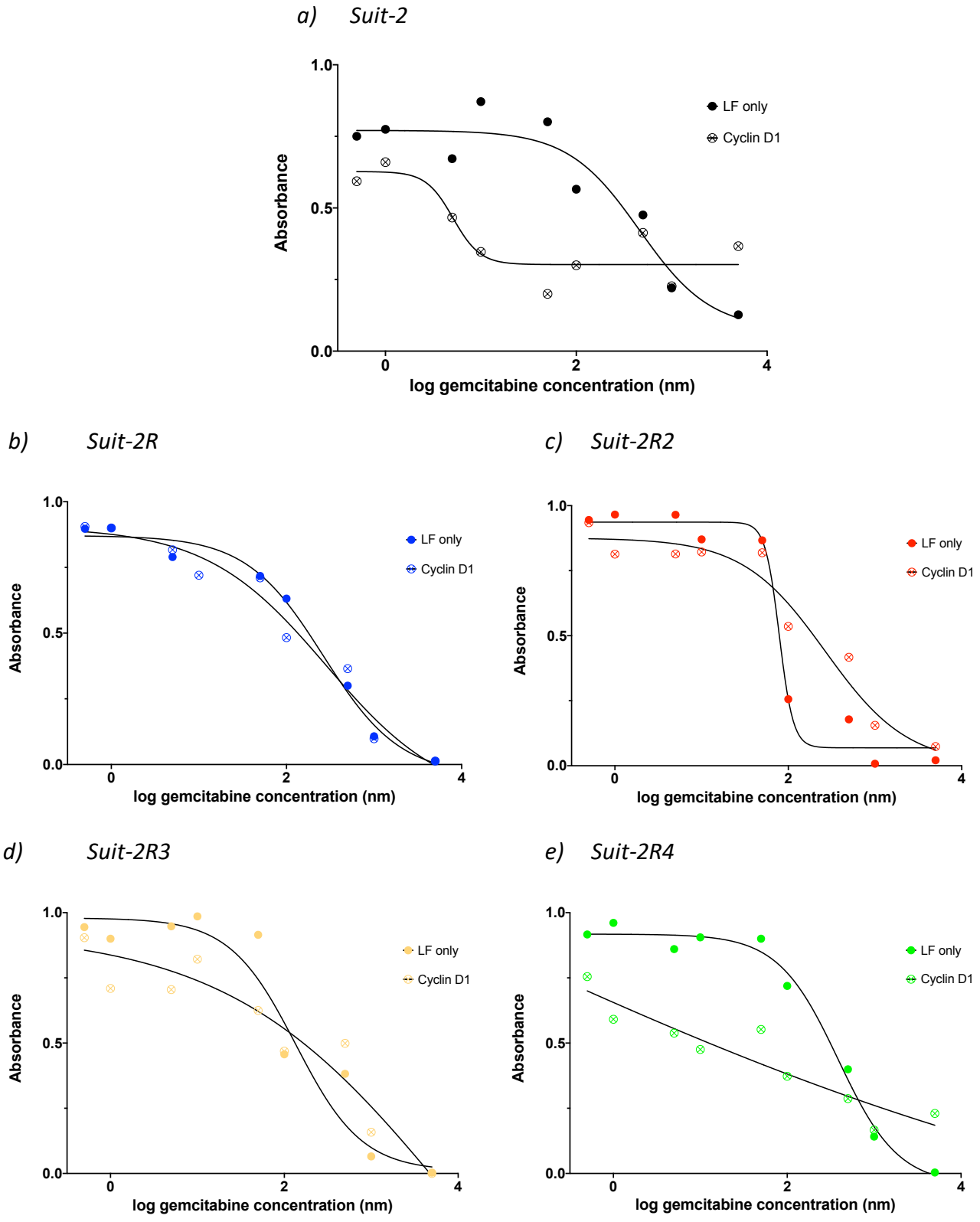


Figure 43 *A cell viability assay of Suit-2 and clonally resistant cell lines Suit-2R, Suit-2R2, Suit-2R3, Suit-2R4 comparing Cyclin D1 knockdown to lipofectamine only control. The absorbance on the y-axis was normalised to a value between zero and one to allow direct comparison of the best fit non-linear regression curve. Suit-2 cells have such a deleterious effect on growth that a drug response curve is not possible, whereas Suit-2R and Suit-2R2 do converge to demonstrate no change to resistance and a possible increase, respectively. Suit-2R3 and Suit-2R4 show a gradual decrease in viability and a drug dose response curve was not feasible at these experimental conditions*

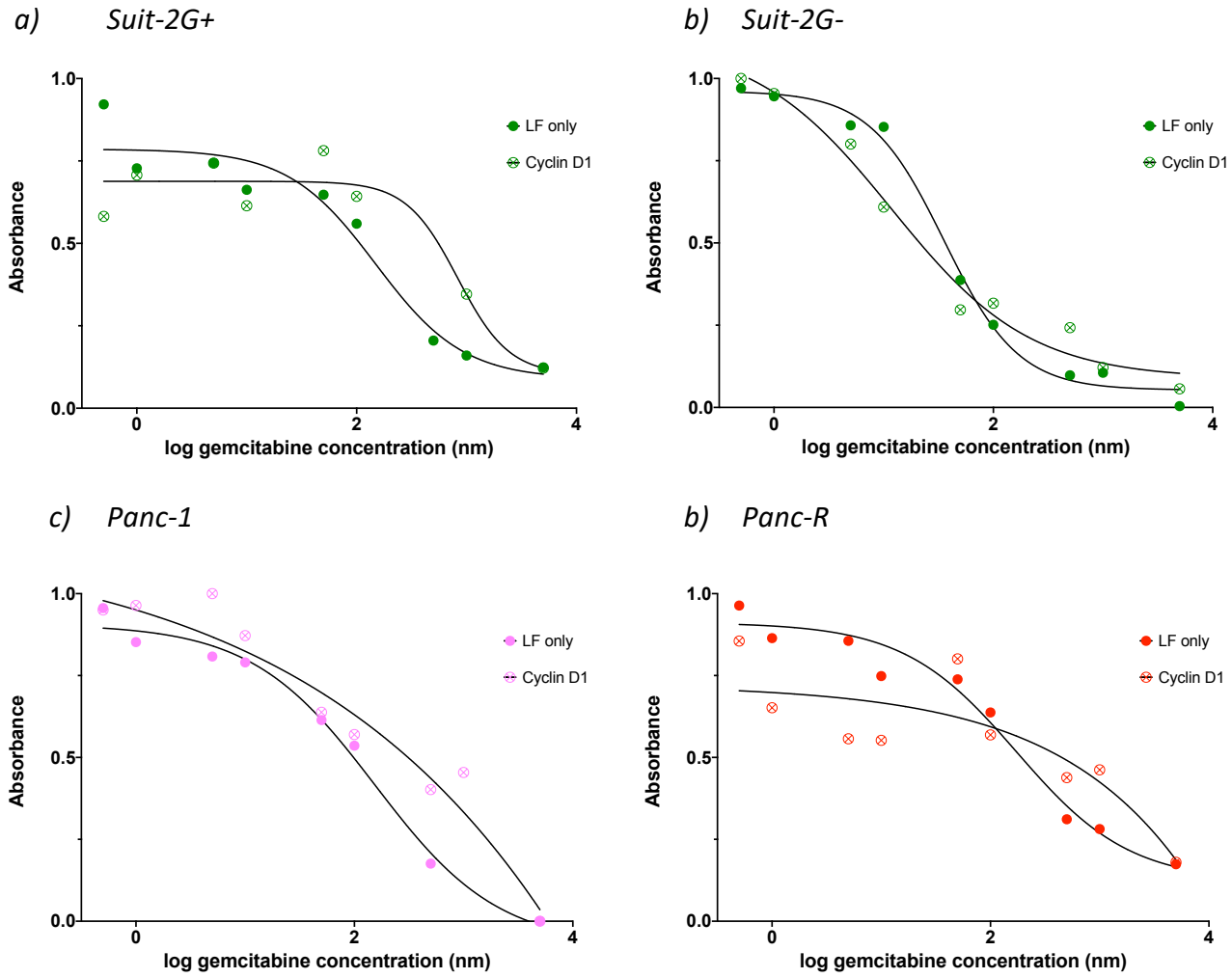


Figure 44 *A cell viability assay of Suit-2G+, Suit-2G-, Panc-1 and Panc-R comparing Cyclin D1 knockdown to lipofectamine only control. The absorbance on the y-axis was normalised to a value between zero and one to allow direct comparison of the best fit non-linear regression curve. Though lipofectamine controls do cause some gemcitabine sensitivity to Suit-2G+ cells, there is a shift in the drug dose response curve to the right indicating increasing resistance. This is not apparent in the Suit-2G- cell line where there may be a small increase to sensitivity. Similar to Suit-2R3 and Suit-2R4 in figure 37, the Panc-1 and Panc-R cell lines demonstrate a general decline in viability at higher doses of gemcitabine without an expected drug dose response curve*

Cell line / Checkpoint marker	**p21** *Tumour suppressor*	**Chk1** *G1/S & G2/M marker*	**CyclinD1** *G1/S checkpoint marker*
Suit-2	-	-	Expressed D1b protein more so than D1a
Suit-2R	Reduced RNA and protein level	Reduced RNA and protein level	Increase RNA levels, D1b expression > D1a Protein levels reduced D1b more prominent
Suit-2R2	Reduced RNA and protein level	Reduced RNA expression, similar protein level to parent	Increase RNA levels D1b expression > D1a D1a absent, D1b level lower than control
Suit-2R3	Low RNA production, similar protein level to parent	Minimal change in RNA expression and increase in protein level compared to parent	Increase RNA levels D1b expression > D1a D1a protein increased compared to control, lower level of D1b expression
Suit-2R4	Reduced RNA and protein level	Increase RNA expression, mild increase in protein level compared to parent	Increase RNA levels, D1b expression > D1a D1a protein increased compared to control, lower level of D1b expression
Suit-2G+	Similar RNA expression to parent, protein level reduced	No change in RNA or protein level	Large increase in D1a, minimal change to D1b protein level
Suit-2G-	Protein level reduced	RNA not performed. No change in protein levels	Large increase in D1a, minimal change to D1b protein level
Panc-1	-	-	Only expresses D1b
Panc-R	Increased RNA and protein level	Reduction in RNA production and mild increase in protein	Only expresses D1b at a lower level to parent Panc-1

Table 8 *Summarising the differences in RNA and protein level to the checkpoint markers p21, Chk1, CyclinD1 in all experimental cell lines.*

There are certain trends that can be described from the observations above:

1. p21 is poorly expressed in all Suit-2 resistant cell lines compared to the parent. Given its importance to overcoming replicative stress, this may present a cells willingness to accept DNA aberrations secondary to gemcitabine. Or there is a lack of active cell cycle division independent of p21.

2. Suit-2R and Suit-2R2 exhibit poor expression of all three markers when compared to Suit-2R3 and Suit-2R4. Suggesting these two lines may have a shared resistance mechanism dependent on a breakdown of checkpoint regulation. Indicating that these cells do not need these checkpoints because the cell is dividing poorly or can survive the damages that require a checkpoint. For Suit-2R3 and Suit-2R4 induction of checkpoints may be occurring normally, indicating they are actively attempting to divide.

3. The Suit-2G+ cell line is dependent on Cyclin D1a to maintain cellular division, especially since p21 and Chk1 are not significantly raised which is expected because dsDNA damage is not increased. Indicating the DDR response is functioning normally in this cell line.

4. Although clonally resistant cell lines over express Cyclin D1a and D1b at an RNA level, this is mitigated at a protein level in all cases. Either through downregulating both D1a and D1b (Suit-2R) or by targeted reduction in D1b relative to D1b (Suit-24). Those cell lines with minimal dsDNA damage from gemcitabine treatment, have high levels of D1a (Suit-2G+ and Suit-2R4) whereas those cells with higher dsDNA damage have reduced cyclin D1a, maintaining a preference for D1b. This switch maybe protective from the Cyclin D1a induced cell cycle arrest, whereas the cell lines that undergo DNA repair efficiently may depend on the Cyclin D1a increase as a stimulus for this process. The siRNA interference experiment shows that Suit-2R and Suit-2R2 may have a protective effect from having low levels of Cyclin D1a.

Gemcitabine resistance is reliant on being able to overcome the effects of replicative stress caused by direct damage to DNA or directly by nucleotide starvation. Gamma irradiation causes DNA damage in all the resistant cell lines with Suit-2G+ having reduced damage compared to parent, whereas all the clonally resistant cell lines have increased damage (most prominent in Suit-2R3). The resistance mechanisms in the clonal cell lines is not the result of prevention/repair of double strand breaks, which is unlikely to be the case with Suit-2G+ cells. The adaptively resistant cells have a massive increase in RRM1 which would prevent nucleotide starvation and allow cell division with consequent risk of double strand breaks. Resistance to these breaks is a logical strategy to

overcome the effect of gemcitabine. Suit-2R and Suit-2R4 has a low level of RRM1 that would be expected to lead to nucleotide starvation which would lead to a reduction in cell division causing replicative stress. The absence of checkpoint markers in Suit-2R indicated that the replicative stress is not being overcome by these regulatory mechanisms, and may indicate that the cell has adapted to cope with continuing levels of stress. In Suit-2R4 these cells are inducing checkpoint markers suggesting a different resistance mechanism in overcoming replicative stress.

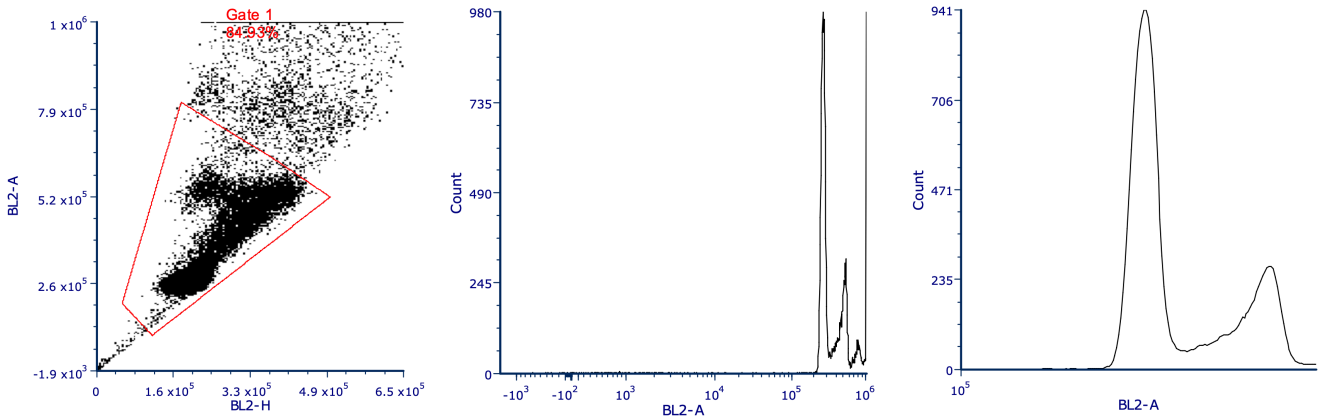
The checkpoint markers p21 and Chk1 are responsible for DNA damage response and regulate transition into the progressive phases. The function of Cyclin D1 is also important here because the G1/S transition specifically depends upon Cyclin D1 induced kinases. Broadly speaking an accumulation of Cyclin D1 pushes the cell cycle into S phase. If we review the cells that have higher Cyclin D1a levels then we can assess those cell that have DNA damage or mitigated DNA damage that push through the G1/S transition, and those that do not. The cells that do not pass this transition enter an arrest phase to allow further DNA repair and await degradation of gemcitabine. This is where Cyclin D1b can play a role as it is less effective as D1a at the G1/S transition, as it is a poor activator of CDKs.

Ultimately final analysis of these markers and collating the cytostatic and cytotoxic analyses required cell cycle analysis, which is the focus of the following section.

3.7 The cell cycle effects of gemcitabine resistance mechanisms

In order to further explore the experiments performed on how ribonucleotide supply, DNA damage and cell cycle checkpoint controls influence gemcitabine resistance, cell cycle analysis is essential. A cell sorting analysis was performed using FACS with the experimental parameters of mean IC50 gemcitabine dose and high dose, similar to above experiments. There were two sets of FACS experiments performed for each cell line with the second set including the supernatant of the culture medium and the cells adherent to the plate surface. This provides a wider perspective on the cell cycle and allows comparison of the cells that detach through mitosis or morbidity or death. There were attempts made at isolating the supernatant separately but this proved difficult as the FACS preparation process causes a degree of substrate loss. Figure 45 shows a representative analysis of a FACS experiment.

a) Cell height vs cell area *b) Frequency histogram* *c) Gated frequency histogram*



d) Cell cycle phased histogram

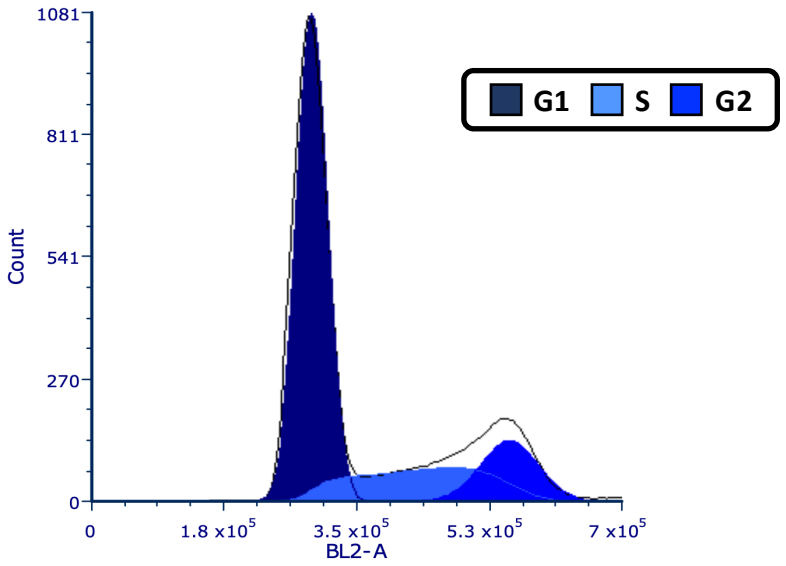


Figure 45 *A representative cell sorting analysis of Suit-2 cells using the BL2 blue laser to detect PI staining: a) Scatter plot of cell height vs area, b) Frequency histogram of all data points, c) Frequency histogram encompassing gating as demonstrated in a), d) Frequency histogram demonstrating cell cycle population using normal distribution modelling, percentages used for following figures*

Figure 46 depicts FACS analysis of all the Suit-2 clonally resistant cell lines and has multiple findings. It is worth noting that Suit-2 cells demonstrate the S phase replication stress bulge (55% at 250nM of gemcitabine) where the cytotoxic and cytostatic effects of gemcitabine prevent successful DNA replication and repair. This leads few cells to enter G2 and subsequently the supernatant contains many cells in G1 and S (91.5%). Suit-2R and Suit-2R2 have a predominant G1 phase at all doses of gemcitabine with no widening of S phase (53.2 and 55% at 250nM respectively). In the attached cells the S-phase, with or without gemcitabine, is relatively small but is greater for all forms of treatments when you include the cells unattached. This suggests that a larger proportion of the dying cells are in S-phase. Consistent with this, there is an increase when you included the supernatant as you increase the dose of gemcitabine. There is potentially a cell cycle arrest to afford DNA repair which explains why the supernatant contains more G1 and S phase cells in Suit-2R (total 97.7% at 250nM gemcitabine). With regards to the supernatant, Suit-2R2 appears to drives cells into G2 at the lower dose of gemcitabine (27.6%) but at the higher dose succumb to S phase non-viability (68.8%). The attached Suit-2R3 cells display a S-phase bulge as the dose of gemcitabine increases (43.3% at 250nM gemcitabine) but when including the supernatant is there is an obvious increase in G2 (14.5% in the control to 33.4% at 250 nM gemcitabine). This suggests cells are pushing through to G2 and into mitosis but struggling to complete cell division. Suit-2R4 cells have the highest proportion of cells in G2 as the dose of gemcitabine increases but, including the supernatant, there are non-viable cells in G1 and S phases. There is a process that sees cells through to G2 successfully but there is more death at the preceding phases. Looking at the distinct resistance mechanisms ascribed Suit-2R and Suit-2R4, in the former cells are remaining viable and arrested in G1, attempts to go beyond G1 result in unattached cells. In Suit-2R4 cell lines, they are able to avoid arrest and enter G2 and the unattached cells appear to accumulate in G1. Remaining in G1 is a survival strategy for Suit-2R but in Suit-2R4 where cell division is maintained, if it fails to progress cells dies in G1 and S. Suit-2R2 seems similar to Suit-2R but Suit-2R3 looks distinct from both Suit-2R and Suit-2R4. In these cells there is replicative stress, as shown by the S-phase bulge, but cells do not appear to be dying as a result. The cells that die appear to have entered G2, perhaps due to mitotic catastrophe. Thus, revealing a potential third resistance mechanism to gemcitabine.

With respect to the Suit-2G cell lines, figure 47 depicts that Suit-2G+ appears to drive cells into G2 with higher doses of gemcitabine with 50.1% of cells in that phase. There is no evidence of replicative stress, and the cells including the supernatant show a diffuse loss cells at all phases, with approximately 36% in G1, 25.5% in S and 38.5% in G2. With Suit-2G- the attached cells show no impact of gemcitabine, without an S phase widening or a G2 increase indicating some cells remain resistant.

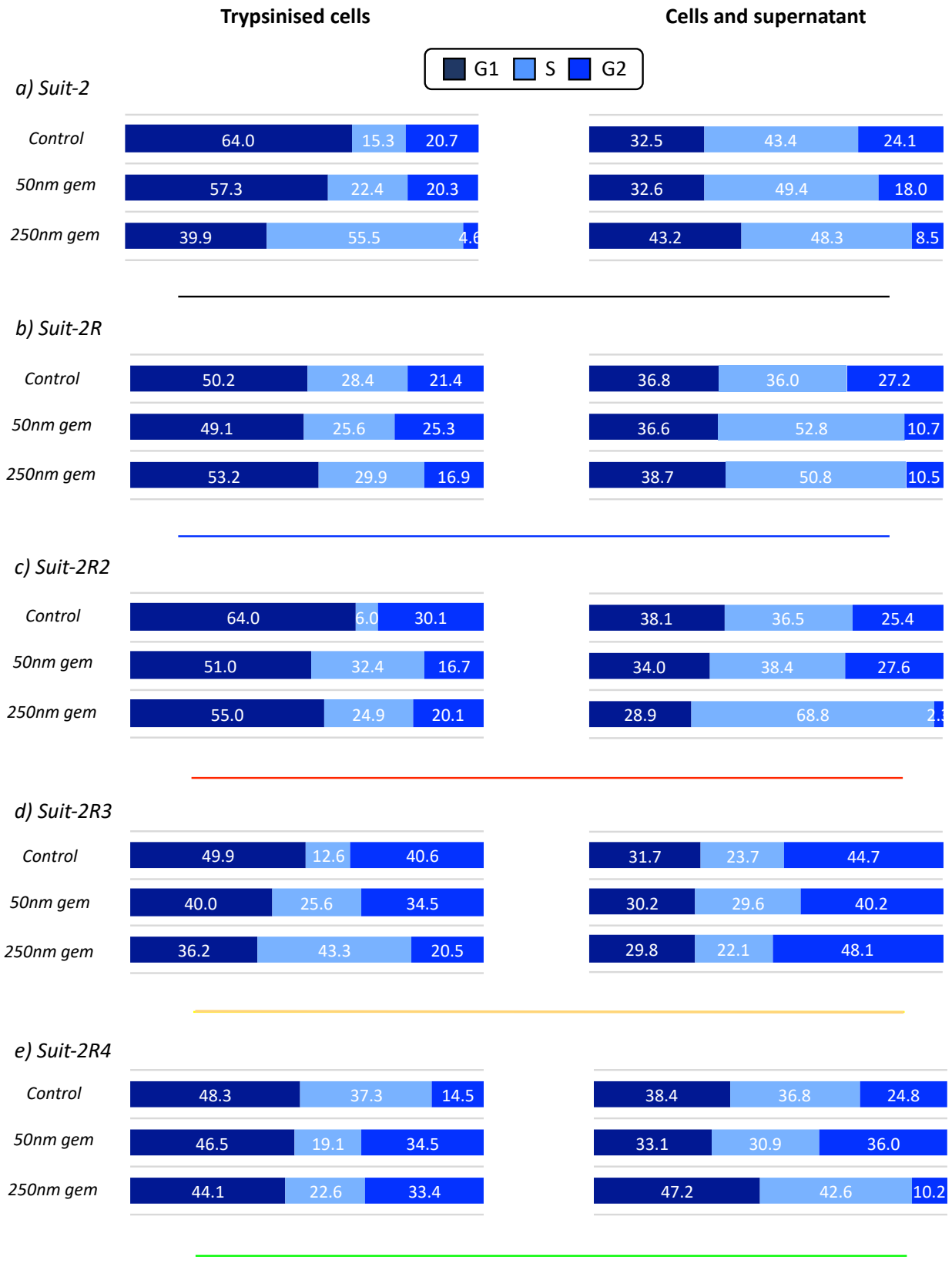


Figure 46 *Horizontal bar graphs depicting cell sorting analysis experiments as cell cycle percentages of a) Suit-2 b) Suit-2R c) Suit-2R2 d) Suit-2R3 e) Suit-2R4 cell lines. On the left were trypsinised cells only and on the right are cells that included the supernatant. Suit-2R & Suit-2R2 cells exhibit no S phase bulge as seen in Suit-2 and Suit-2R3 and 53.2% and 55% of cells remain in G1 respectively. There are cells that have sloughed off in the supernatant at higher doses of gemcitabine and few cells enter into G2 (10.5% and 2.3% respectively). Suit-2R3 exhibits a widening of the S phase but more cells pass through to G2, appropriately*

or not and this is seen as the cells that in the supernatant are predominately G2. Suit-2R4 cell lines have the lowest proportion of cells in S phase and the highest in G2 at 250nM of gemcitabine indicating that cells are entered into G2 rapidly, and the deatched cells succumb to death in earlier phases.

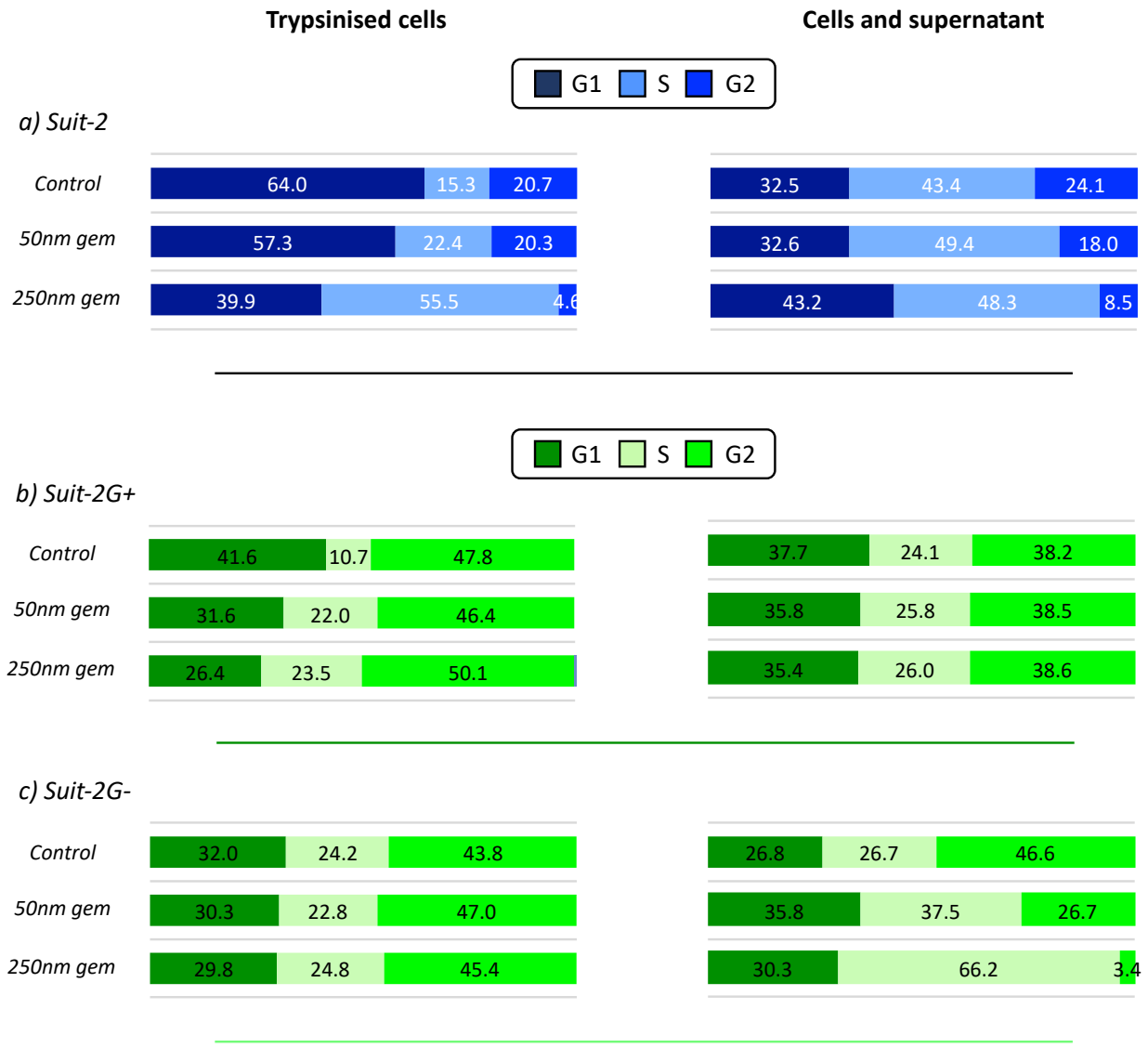


Figure 47 *Horizontal bar graphs depicting cell sorting analysis experiments as cell cycle percentages of a) Suit-2 b) Suit-2G+ and c) Suit-2G-. On the left were trypsinised cells only and on the right are cells that included the supernatant. Neither Suit-2G cell exhibited the S phase bulge, and Suit-2G+ demonstrates a progression through the cell cycle with higher proportions in S and G2 at higher doses of gemcitabine. There is a consistency in the supernatant at all cell lines, with no effect with gemcitabine. Suit-2G- cells exhibit similar stability with almost equal cell cycles proportions but when including detached cells, there is S-phase death. These cells have to to return to sensitivity.*

When including the supernatant, there is a significant number of cells that have lost this resistance and are dying as typical in S-phase (66.2%) with few in G2 phase (3.4%). Suit-2G- has not completed its journey to sensitivity and there are heterogenous populations of cells that are resistant or undergo replicative stress and are shed into the supernatant.

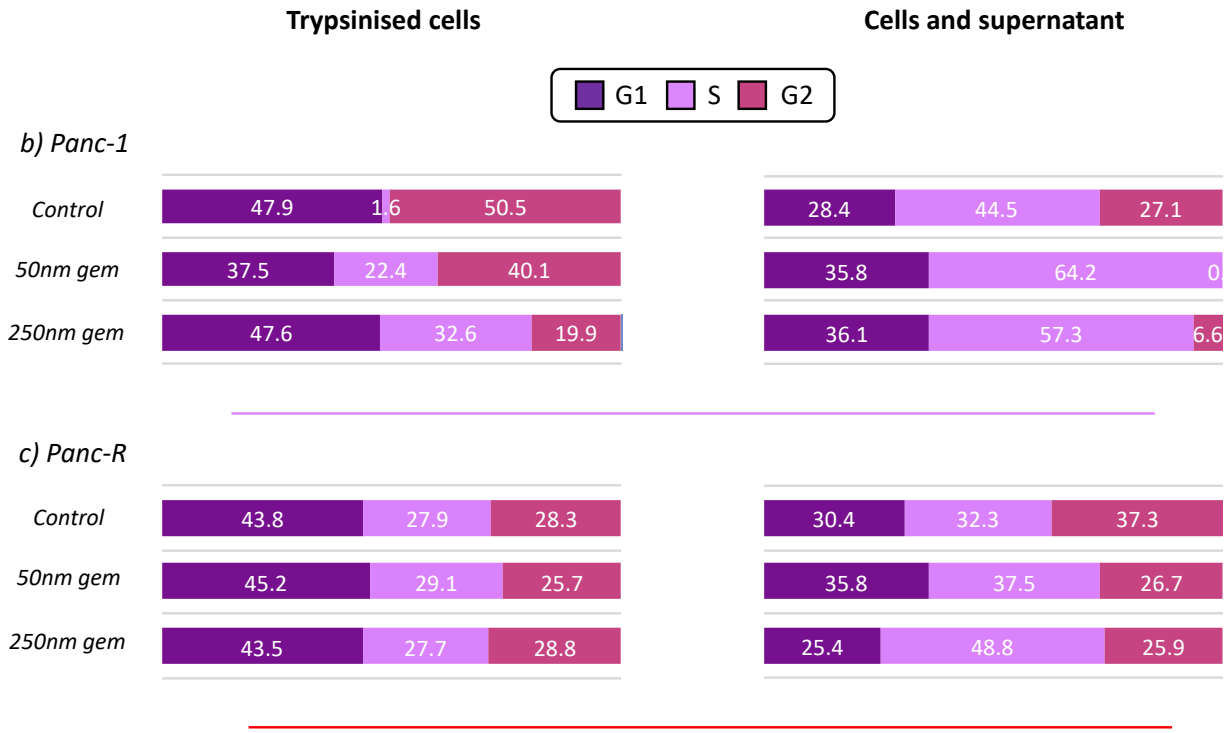


Figure 48 *Horizontal bar graphs depicting cell sorting analysis experiments as cell cycle percentages of a) Panc-1 and b) Panc-R. On the left were cultured cells with culture medium discarded and on the right are cells that included the supernatant. The Panc-1 cell lines demonstrates the widening S phase as with Suit-2 and sees cells sloughing off in predominately S phase, as expected with the effects of gemcitabine though prefers to progress to G2 with 50.5% at control. There is remarkable stability with Panc-R cells at all parameters at the base of the plate, though cells fail to survive in S-phase when combining the supernatant (27.7% vs 48.8%) . There is a drive to leave S-phase and push into G2, despite the risk of death*

Figure 48 demonstrates the Panc-1 cell lines which does exhibit the S phase bulge from 1.6% to 32.6%, control to 250nM of gemcitabine in the parent line. Even in the absence of gemcitabine, in contrast to Suit-2, there are very few cells in S-phase. Most that are in this phase have detached from the plate. When combining the supernatant there are high loses at G1 and S phases with a total proportion of 100% and 93.4% at 50nM and 250 nM of gemcitabine respectively. This implies that cells are unhappy in S-phase and sensitive to replicative stress. Panc-R maintains a stable cell cycle throughout treatment of gemcitabine with approximately 44% in G1, 28% in S phase and 28% in G2, with cells attached to the plate. This implies an ability to survive in S-phase compared to the sensitive parent. Combining the supernatant, at 50nM of gemcitabine there begins an increase in G1 and S phase non-viability with 35.8% and 37.5% respectively and then at 250nM more cells slough off in S phase (48.8%). Although there is stability in the attached cells, there are a lot of cells trapped in S phase as the dose of gemcitabine increases. Panc-R tolerates replicative stress like Suit-2R4 in comparison to its parent strain, but there is evidence that Panc-1 is more resistant to gemcitabine that Suit-2.

The resistance mechanisms can be loosely divided into the ability to tolerate replicative stress and an ability to avoid replicative stress. Suit-2R is an example of avoiding replicative stress with an accumulation of cells in G1. Suit-2R4 is archetypal of a cell line that tolerates replicate stress with large proportions of cells in S phase progressing to G2. Suit-2R3 is another example of a cell line tolerating replicative stress, but in this case has higher non-viability as it enters G2/M. It completes DNA replication but suffers the consequence of replicative stress as the cell tries to divide. There are fewer cells in G2 overall when considering the supernatant suggesting that cell death occurs earlier in the cell cycle because of gemcitabine induced stress replication.

There are clearly significant differences in the behaviour of these cell lines at a cell cycle perspective and a summary of all experiments performed in all cells lines is show in Table 9.

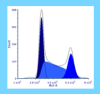
		Suit-2	Suit-2R	Suit-2R2	Suit-2R3	Suit-2R4	Suit-2G+	Suit-2G-	Panc-1	Panc-R
Resistance	Gemcitabine IC50	10.1nM	24.3nM	22.8nM	86.7nM	52.7nM	Unrecordably high	12nM	15.4nM	61.7nM
	Growth rate of cells	—	Slow growth	Normal growth	Normal/Fast growth	Normal/Fast growth	Normal growth	Normal growth	—	Normal growth
Cytotoxic	dsDNA with control	Basal — 8Gy —	Basal ↔ 8Gy ↔	Basal ↓ 8Gy ↑	Basal ↑ 8Gy ↑	Basal ↓ 8Gy ↑↑	Basal ↔ 8Gy ↓	Basal ↓ 8Gy ↓↓↓	Basal — 8Gy —	Basal ↔ 8Gy ↔
	dsDNA with Gemcitabine	50nM — 250nM —	50nM ↓↓ 250nM ↓↓	50nM ↔ 250nM ↑	50nM ↓↓ 250nM ↔	50nM ↔ 250nM ↓	50nM ↓↓↓ 250nM ↓↓	50nM ↔ 250nM ↓	50nM — 250nM —	50nM ↔ 250nM ↓↓
Cytostatic	Ribonucleotide reductase complex	RNA Protein RRM1 — — RRM2 — — p53R2 — —	RNA Protein RRM1 ↔ ↓ RRM2 ↔ ↓ p53R2 ↔ ↔	RNA Protein RRM1 ↓ ↑ RRM2 ↓ ↔ p53R2 ↓ ↔	RNA Protein RRM1 ↓ ↑ RRM2 ↓ ↔ p53R2 ↓ ↔	RNA Protein RRM1 ↓ ↓ RRM2 ↑ ↔ p53R2 ↓ ↓	RNA Protein RRM1 ↑↑↑ ↑↑↑ RRM2 ↔ ↔ p53R2 ↓ ↔	RNA Protein RRM1 ↑↑ ↑ RRM2 ↔ ↔ p53R2 ↓ ↔	RNA Protein RRM1 — — RRM2 — — p53R2 — —	RNA Protein RRM1 ↑ ↑ RRM2 ↓ ↑ p53R2 ↓ ↔
	RRM1 • Gem effect • Knockdown • Transfection	Gem Tx ↑50 ↓250 Growth IC50 KD ↓ ↓ TF ↑ ↔	Gem Tx ↑50 ↓250 Growth IC50 KD ↓ ↔ TF ↓ ↔	Gem Tx ↑50 ↓250 Growth IC50 KD ↓ ↔ TF ↔ ↔	Gem Tx ↓50 ↓250 Growth IC50 KD ↓ ↔ TF ↓ ↔	Gem Tx ↑50 ↑250 Growth IC50 KD ↓ ↑ TF ↓ ↔	Gem Tx ↑50 ↓250 Growth IC50 KD ↔ ↓ TF ↓ ↔	Gem Tx ↑50 ↓250 Growth IC50 KD ↓ ↔ TF ↓ ↔	Gem Tx ↑50 ↓250 Growth IC50 KD ↓ ↔ TF ↓ ↔	Gem Tx ↑50 ↑250 Growth IC50 KD ↓ ↔ TF ↔ ↔
Checkpoint markers	Cyclin D1a&b G1 regulator	RNA Protein CycD1a — — CycD1b — — KD Growth ↓ More sensitive to gem	RNA Protein CycD1a ↑ ↓↓ CycD1b ↑↑ ↓ KD No change to growth or resistance	RNA Protein CycD1a ↑ ↓↓ CycD1b ↑↑ ↓ KD Growth ↓ IC50 ↑	RNA Protein CycD1a ↑ ↑ CycD1b ↑↑ ↓ KD Growth ↓ No real IC50 change	RNA Protein CycD1a ↑ ↑↑ CycD1b ↑↑ ↑ KD Growth ↓ & likely more sensitive	RNA Protein CycD1a ↑↑↑ CycD1b ↔ KD Growth ↓ IC50 ↑	RNA Protein CycD1a ↑↑↑ CycD1b ↔ KD Growth ↓ IC50 ↓	RNA Protein CycD1a 0 CycD1b — KD Growth ↓ & No real IC50 change	RNA Protein CycD1a 0 CycD1b ↓ KD Growth ↓ & No real IC50 change
	p21 G1 suppressor	RNA – Protein –	RNA ↓ Protein ↓	RNA ↓ Protein ↓	RNA ↓ Protein ↔	RNA ↓ Protein ↓	RNA ↔ Protein ↓	Protein ↓	RNA – Protein –	RNA ↑ Protein ↑
	Chk1 Checkpoint marker	RNA – Protein –	RNA ↓ Protein ↓	RNA ↓ Protein ↔	RNA ↔ Protein ↑	RNA ↑ Protein ↑	RNA – Protein –	Protein –	RNA – Protein –	RNA ↓ Protein ↑
Cell cycle	FACS analysis 	S phase bulge with gemcitabine treatment, fewer cells progressing to G2 with supernatant	Cells stall mainly in G1, widening, though non viable cells consistent across all phases with supernatant	Cells predominately in G1 but driven to G2, accepting death in S phase as gemcitabine dose increases	S phase bulge is present but cells are driven into G2, accepting aberrant DNA replication	Cell progress to G2 avoiding an arrest, with unattached cells accumulating in G1, unable to survive	Gem stimulates progression into G2, non-viability spread out & consistent at all doses	Stable transitions at all gemcitabine doses but not effect of gemcitabine. Not fully returned to sensitivity	Does develop the S phase bulge but at control few cells in S phase. Sensitive to replicative stress	Maintains a stable cell cycle with an ability to survive in S-phase, though still detach in that phase

Table 9 *Summarising all experimental findings in all resistant and parent pancreatic cancer cell lines*

4. Discussion

Chemotherapy treatment of pancreatic cancer has only recently progressed from a single drug regimen to either coupled or multiple combination therapies with gemcitabine or 5-fluorouracil (5-FU) as their base. These traditional chemotherapy agents remain the foundation of treatment in pancreatic cancer. Clinical trials have proven the increased effectiveness and highlighted the particular need for biomarker detection. The 5-FU based four drug FOLFIRINOX regime is a broad-spectrum solution that brings with it a wide spectrum of toxicity complications. For this reason, the elderly and co-morbid populations are still receiving single agent regimens of gemcitabine or dual combination coupled with capecitabine. There have been high reported rates of gemcitabine resistance and given the increase in chemotherapeutic options, a more tailored approach would allow patients to receive the most efficacious therapy and avoid the most deleterious to their health (5, 27). Investigating the resistance mechanisms of the tumour could allow a personalised chemotherapy approach by identifying which patients are likely to develop resistance and possibly identify methods to overcome this. In this thesis, evidence has been presented that there is not just one resistance pathway, and that resistance can be achieved through very different strategies. All of which need to be properly understood in order to identify biomarkers appropriately. For example, high and low levels of ribonucleotide reductase can be selected for, to give resistance in different individuals.

4.1 Resistance generation

In researching the methodologies of developing gemcitabine resistant pancreatic cancer cells, the relatability to a clinical chemotherapy drug regimen was a pertinent consideration. The exposure of established pancreatic cancer cells to progressively increasing doses of gemcitabine does not relate to clinical practice. Patients receive one dose of gemcitabine once a week for two to three weeks and then further doses for a tailored number of cycle regimens, or until the patient is unable to tolerate the side-effects any longer. Therefore, the exposure of a pancreatic tumour to a chemotherapy drug is not at a consistent level and not for a constant period of time. This is a pulsed regimen and any *in vitro* experiments need to reflect that process for conclusions to be translatable.

Panc-1 and Suit-2 immortalised pancreatic cancer cell lines are sensitive to gemcitabine, though the former has a higher degree of resistance, as shown by an IC50 of 15.4nM compared to 10.1nM. In order to develop resistance, a process is required to select populations of cells that are resistant to

gemcitabine. Two methods were employed; clonal selection or increasing dose exposure as adaptive strategies to resistance. The single cells that survive exposure to gemcitabine develop mechanisms to enable DNA replications and mitosis, or have innate characteristics that allow cell growth. When the clonally isolated resistant cell lines were developed, strategies to pulse gemcitabine on a more frequent basis resulted in widespread death. This was because cells were unable to compensate for the effects of the drug or degrade it quickly enough. Surviving clonal islands only consisted of a few cells on a 96-well plate. Whereas adding further gemcitabine to a T75 flask of Suit-2 cells afforded many cells of varying innate functionality to manage the cytotoxic and cytostatic effects. This self-selects a heterogenous population in comparison to those cells that are clonally isolated. Once gemcitabine is degraded by a sub-population of cells in a heterogenous population, other cells will avoid replicative stress because nucleotide levels will be maintained. Once gemcitabine is removed from culture media then the non-resistant cells are able to grow freely and the population will become sensitive. Whereas the single isolated cell undergoes mitosis as the only gemcitabine surviving clone, giving a population of resistant cells. Clearly there are implications to the development of both resistance strategies that will impact on their clinical significance. These will be discussed after the resistance mechanisms have been considered.

4.2 Fundamentals of gemcitabine resistance

The fundamentals of gemcitabine toxicity stem from the induction of stress to the DNA replicative phase, directly through incorporation into DNA and indirectly by obstructing the production of nucleotides. Replicative stress results from an inability for a cell to undergo DNA synthesis in the absence of a process to stop replication. DNA damage which induces cell cycle arrest therefore does not cause replicative stress (DNA damage that does not cause cell cycle arrest will cause replicative stress by definition). In its very nature, it induces cell cycle death by preventing division and interfering with normal cell division processes. In order for a pancreatic cancer cell to survive this insult, it has to either prevent entry of gemcitabine or compensate/negate its effects. In order for a cell to compensate for the effects of gemcitabine, survival will involve regulation of cell cycle progression. Either by arresting the cell cycle to allow DNA repair processes or to pushing through replication at the cost of damaged DNA which somehow the cell manages to survive. The premise that resistance can either result from avoiding DNA damage or by tolerating DNA damage underlies this thesis. The experimental findings of DNA damage, ribonucleotide reductase alterations and adjusted cell cycle checkpoint markers all combine to explain how cell cycle regulation may increase the chance of cell survival.

4.2.1 Adaptive resistance

The Suit-2G+ cell line appears to have a clear resistance process with the overwhelming increase in RRM1 acting as its primary focus. The increased production of ribonucleotide reductase could counter the toxic effects of gemcitabine allowing the dilution of dFdCDP by unmodified deoxynucleotides. There is no increase in RRM2 or p53R2 explained by the fact that RRM1 is bound by gemcitabine. The production of a functional RR complex may not only obviate the cytostatic effect of gemcitabine but allow the increase of the foundation blocks for DNA replication. We only see double stranded DNA damage at higher concentrations of gemcitabine and even then, it is still significantly less than with the Suit-2 cell lines. This may be explained by a greater proportion of RRM1 being free of dFdCDP giving a greater production of the non-cytotoxic dCTP (which will compete with the fluorinated nucleotide reducing DNA damage). There is some confirmation of this process by the fact that RRM1 increases when Suit-2G+ cell lines are treated with gemcitabine as seen in figure 24b. However, the RRM1 siRNA interference process does not seem to affect resistance, indicating that once resistance has been established the requirement for high levels of RRM1 may no longer absolute. In Suit-2G+ cells both p21 and Chk1 are low in expression indicating that DNA damage is not inducing cell cycle arrest, but may be causing replicative stress. Hence why Cyclin D1a is accumulated in these cells. The cell functions with high efficiency which is explained by the FACS analysis, where the only changes seen are an increase through to G2 and consistency with the supernatant. Quite simply gemcitabine stimulates this cell into rapid and effective growth which is also the reason why an IC50 cannot be produced, a growth reduction in 50% is not achieved even at the highest concentrations employed. The initial resistance development process selected cells that were able to produce high amounts of RRM1 and they continued to select and divide at higher doses of gemcitabine.

Further support of the Suit-2G+ theory are the experimental findings of Suit-2G-. The removal of a gemcitabine stimulus returned these cells to a normal sensitivity with an IC50 of 12nM but the effects of once being Suit-2G+ cells still linger. There is still a high level of RRM1 protein and significantly increased DNA damage, as seen in Suit-2 cells. There may not be enough of an increase in RRM1 expression to compensate for the cytotoxic damage caused by gemcitabine, alternatively other changes necessary for resistance found in Suit-2G+ may have been lost. The cell cycle checkpoint markers still remain similar to the Suit-2G+ cell line with low levels of p21 and Chk1 and high CyclinD1a expression. The cells have adapted to a rapidly transitioning cell cycle but are succumbing to stress replication and cell death in S phase in the presence of gemcitabine. This is seen in the cell cycle analysis where cells are progressing into S/G2 but are non-viable, as they detach into the

supernatant. In fact, just reviewing the cells adherent to the plate, there is almost no effect of gemcitabine. These cells have lost resistance but the resistance mechanisms in place still apply to the sub-population that managed to survive in the presence of gemcitabine. When CyclinD1 is knocked out, the cell cycle is inhibited and the sensitivity to gemcitabine increases. Presumably because most cells are unable to drive into the mitotic process and those that are, die during in S phase. The Suit-2G cell lines require gemcitabine exposure to maintain resistance to it and there is a threshold when the increase in RRM1 expression is sufficient to overcome the bimodal toxicity of gemcitabine.

4.2.2 Clonal resistance

The clonal isolation cell lines remain resistant without gemcitabine, albeit at significantly lower levels compared to Suit-G+. The lowest IC50s belong to Suit-2R and Suit-2R2 (24.3nM and 22.8nM respectively) and the highest to Suit-2R3, Suit-2R4 and Panc-R (86.7nM, 52.7nM and 61.7nM respectively). The difference between the IC50s in Suit-2R/Suit-2R2 and suit-2R3/Suit-2R4 already indicated potential differences in resistance mechanisms. The lower IC50s in comparison to Suit-2G+ may be explained in their culturing maintenance. With respect to Suit-2G+ cells, a position has been reached where gemcitabine is totally destroyed or prevented from entering the cells at all. This state is maintained by the presence of gemcitabine. This is analogous to an individual who maintains lactose tolerance while they are exposed to dairy products but when dairy is removed, an individual may gradually over time become lactose intolerant. In the clonal cells this is more analogous to individuals who are lactose intolerant due to inherited mutations and in order to become tolerant would require further mutation. These are cell lines that grew from a mutated single cell that was able to multiply in cell culture infused with gemcitabine. There was no further cell selection except at that initial single cell level. A clonal isolation cell line grown without gemcitabine in conjunction with clonal cells grown with increasing doses, would have provided a more relevant control that the parent Suit-2 cell line, this was not carried out at the beginning of the resistance generation process.

It must be considered that the delivery of the active gemcitabine metabolite, into the cell may be altered, and though there were no obvious changes to CDA or DCK discovered, the possibility of hENT1 expression alterations cannot be ruled out. However, in this thesis, it has demonstrated that regardless of any change to influx there are changes in the level of gemcitabine target in all the resistance cell lines. Looking further into the metabolism of gemcitabine and given the prominent role of RRM1 in the Suit-2G+ cell line, it is a pertinent starting point to try and describe the delicate balance between DNA replicative stress and cell cycle progression.

The five clonally resistant cell lines can also be divided into two groups based upon their expression of RRM1 compared to the parent cell line: Suit-2R and Suit-2R4 have reduced expression whereas Suit-2R2, Suit-2R3 and Panc-R have a high expression. The cell lines can be further divided on relationship between RNA and RMM1 protein levels. In the Suit-2G+ cell line the effect is clear whereas there is no relationship in the Suit-2R/Panc-R cell lines suggesting that the level is altered post-transcriptionally. This also may explain that except for possibly Suit-2R4, the modifications to RRM1, whether by siRNA interference or heterologous expression, fail to alter resistance to gemcitabine. Though it has been shown in these two experiments that RRM1 expression can be changed, all it effectively does is alter the growth rates, which is to be expected. Therefore, there is likely to be a delicate balance that determines how the cell manages replicative stress and the cell cycle, through regulation of RRM1.

4.2.2.1 Low RRM1

Suit-2R and Suit-2R4 have reduced RRM1 expression and when observing the proportion of dsDNA damage, they share a common trait: a significant reduction in DNA damage compared to the parent Suit-2 line in the presence of gemcitabine. When treated with gemcitabine, there must be mitigating measures to prevent significant replicative DNA damage, this may be in the form of ATM or ATR pathways that recruit downstream repair kinases. Whatever the repair mechanisms, we can be confident there are fundamental differences between Suit-2R and Suit-2R4 by reviewing the basal and radiation controls. In Suit-2R, basal levels and induced levels of DNA damage from gamma radiation seem equivalent to the parent cell line, whilst in Suit-2R4 basal levels of dsDNA damage seem particularly low but they seem to be more sensitive, not less, to gamma radiation. It can be concluded that low RRM1 expression is associated with reduced gemcitabine induced dsDNA damage, but this may not be through a general pathway protecting against DNA breaks. It appears to be through at least different mechanisms.

Suit-2R and Suit-2R4 exhibit further differences, especially in resistance levels as one is high and one is low but also one grows significantly faster. Cell cycle analysis also demonstrate divergent attributes. Suit-2R have a large proportion of cells in G1 and do not exhibit an S phase widening in the presence of gemcitabine, which would be expected with replicative stress induced by the drug. Suit-2R4 has more cells in G2 than G1 phases and also does not have a large S-phase component in the presence of gemcitabine, in fact the S-phase component actually decreases in detached Suit-2R4 cells. Suit-2R contain the fewest cells in G2 of any of the clonal cells observed. Those cells that have detached from the plate (mitotic death) have a large S-phase component. Suit-2R4 cells that have

detached demonstrate an even distribution at all phases at 50nM of gemcitabine, and at 250nM the S-phase, although significant, is not as large as in Suit-2R.

In summary, Suit-2R cells have viable cells in G1 and, in comparison to Suit-2R4, less cells have progressed in to G2. The dying Suit-2R cells seems to be mainly in S-phase whilst a greater proportion of Suit-2R4 cells are in G1 (having failed to enter S-phase at all) at the point of death.

The checkpoint regulators p21 and Chk1 will be upregulated when a cell enters cell cycle arrest between G1/S or G2/M. Arrest contrasts with a cessation of growth, in being an active process. The expression of p21 and Chk1 levels in Suit-2R are considerably lower than was seen in the parent cell line. Combining this with the fact that Suit-2R is growing slowly, and that this cell line has a reduced level of ribonucleotide reductase, it indicates that this could be a modification to avoid cell cycle arrest and instead maintain a slow and measured cell division (perhaps pausing rather than arresting before entering S phase). It is notable that the majority of Suit-2R cells are in G1, suggesting that Suit-2R delays the G1/S transition without arresting. Given the low level of RRM1 it is reasonable to assume that this is because of limiting levels of nucleotides. The problem for the Suit-2R cells would be the high level of assumed replicative stress and a possible compensatory mechanism may be the downregulation, post-transcriptionally, of Cyclin D1, most significantly D1a. These cells can therefore be predicted to have a very low level of CDK4-driven S-phase entry, reducing replicative stress.

Once again in contrast to Suit-2R, Suit-2R4 shows an elevated level of Chk1, which maybe causing checkpoint induction between G2/M as noted on cell cycle analysis. p21 is downregulated suggesting that checkpoint control is not as active as seen in the parent strain and it could be hypothesized that the low level is matched by an increased passage through the G1/S checkpoint. Given the low level of RRM1, this is likely to result in replicative stress but Cyclin D1 is elevated, as opposed to reduced as seen in Suit-2R. It can therefore be assumed there is a drive through S-phase with a high level of replicative stress and there is no obvious compensation mechanism involving Cyclin D1. Not only are the levels of Cyclin D1 high (increase levels of CDK activity), the most stress inducing isoform (D1a) is predominantly expressed. Once it passes S-phase, the cell then pauses or arrests in G2/M.

4.2.2.1 High RRM1

Suit-2R2 and Suit-2R3 have high RRM1 expression, suggesting excess levels of ribonucleotide reductase compensating for the inhibition by gemcitabine. Hence, they are able to supply nucleotides required for DNA replication and avoid this element of replicative stress. This appears to be a post-transcriptional increase, as the RNA levels are less than the parent in both Suit-2R2 and Suit-2R3. Reviewing this in combination with the dsDNA damage, Suit-2R2 has reduced damage at a basal level and at 50nM of gemcitabine treatment, as would be expected with increased nucleotides, whereas Suit-2R3 has higher basal dsDNA breaks with reduced damage at 50nM gemcitabine. This is indictive of more efficient management of gemcitabine induced DNA damage. Both cell lines have increased damage at 8Gy radiation implying that there is not an improvement in overall DNA repair to explain gemcitabine resistance. Although, the increased supply of nucleotides in the presence of gemcitabine may be a pertinent fact in the management of DNA damage. Given that the high IC50 for Suit-2R3 implies it has the greater ability to manage gemcitabine induced dsDNA breaks, although at very high levels of the drug this appears to breakdown. The reduced level of RRM1 in Suit-2R3 at high doses of gemcitabine treatment, may starve this cell line of nucleotides, preventing entry into S phase. While for Suit-2R2 these cells enter S phase to suffer DNA damage and replicative stress.

There is a variation in how these cells respond to gemcitabine exposure, Suit-2R2 increases expression of RRM1 and Suit-2R3 reduces it. It is difficult to know at what thresholds each cell has regarding how to balance RRM1 production to compensate the cytostatic and cytotoxic effects of gemcitabine. In either case, it affects the DNA damage that can be sustained and repaired and will reflect how the cell cycle is controlled.

Suit-2R3 depicts a widening of the S-phase as gemcitabine increases and is the only cell to demonstrate this outside of the control cell lines. This reflects the replicative stress the cell is under due to gemcitabine, despite it being more resistant than the controls. These cells are still progressing to G2 and those adherent to the plate have 43% in S phase but almost 21% are in G2. There is a marked difference in combining cells in the supernatant because G1 and S phase death is not seen, at 250nM of gemcitabine, almost half the cells are in G2. This G2 predominance is not seen as significantly in any other cell line. These cells appear willing to undergo replicative stress, and this may be a feature even in the absence of gemcitabine, which could explain why at the basal there are more dsDNA breaks than in control cells. They are willing to continue through S-phase in the presence of double strand breaks. Suit-2R2 cells have the opposite effect, as over half the attached cells are in G1 suggesting they are not willing to undergo replicative stress. The cells that included with the

supernatant demonstrate widespread death at G1 and S, especially at the higher dose of gemcitabine. It can be stated that these two cell lines have differing strategies in overcoming replicative stress, just as Suit-2R and Suit-2R4 have different strategies: Suit-2R2 pauses the cell cycle to prevent replicative stress and Suit-2R3 accepts it and drives into G2 at the cost of abnormal DNA replication possibly leading to inappropriate mitosis.

Suit-2R3 has a high level of ribonucleotide reductase and therefore less supply constraints to the nucleotide levels, improving its ability to undergo replication. The most notable checkpoint changes are that p21 levels are equivalent to the parent strain and Chk1 levels are upregulated. This cell line may maintain an ability for G2/M cell cycle arrest to avoid damage from gemcitabine. Checkpoint control in this case seems to protect the cell and as with Suit-2R4, stress induction is increased, but instead is compensated for by an increased supply of nucleotides. Suit-2R2 which also has a high level of ribonucleotide reductase, has a reduction in p21 suggesting deficiency in checkpoint control. This reflects the reduction in the expression of the most stress inducing isoform of Cyclin D1; D1a.

Panc-R is similar to Suit-2R2 and Suit-2R3 with high expression of RRM1, but unlike those cell lines it correlates with a higher RNA transcript production. In that respect it is similar to the Suit-2G+ cell line which is also has increased RRM1 expression at the RNA level. This cell lines also sustains less dsDNA damage, especially at 250nM of gemcitabine treatment compared to the Panc-1 cell line, with similar levels at radiation controls. From the perspective of the cell cycle, the viable cells show stability of phases with stable S-phase at all doses of gemcitabine. When the supernatant is included, it is clear that there is death in all phases of the cell cycle. Therefore, the Panc-R cell line in common with Suit-2R3 and Suit-2R4, does not pause before S-phase in order to avoid replicative stress and DNA damage. In contrast to Suit-2R3 and Suit-2R4, Panc-R may restrict cell cycle transition through checkpoints (elevated p21 levels) and reduced drive into S-phase (reduced Cyclin D1, with the less stress inducing Cyclin D1b). Panc-1 cells exhibit a homozygous phenotype at the G870A SNP, where there is a G allele (Figure 37). This nucleotide position affects the frequency of splicing of CyclinD1 resulting in more of the D1b variant. This variant appears to be related to tumourigenesis and lower replicative stress. The Panc-R cell line further reduces stress by lowering the total level of Cyclin D1 levels. It is not surprising therefore, that no gemcitabine sensitivity develops from further knockdown of the protein.

4.3 Summary

In the generation of the aims of this thesis, it was assumed that resistance to gemcitabine could be determined genetically in sub-clones of cancer cells, which could then be selected for. Furthermore, it was assumed that another way to reach a state of resistance would be for cells to adapt in the presence of gemcitabine. It was not clear whether this represented two distinct survival mechanisms or whether this was two methods of reaching the same destination. In addressing these aims, a series of hypotheses were based on the known action of gemcitabine. The phosphorylation process, the cytotoxic and cytostatic effects all require investigation before trying to understand how a pancreatic cancer cell survives treatment with gemcitabine. In the presence of gemcitabine, a survival strategy would be to increase the level of ribonucleotide reductase activity and/or increase the capacity for DNA repair. It is well established that stress of any type can lead to changes in expression that are maintained during that period and for a short time afterwards. The observation that RRM1 increased dramatically in cells exposed to gemcitabine was therefore not surprising. The question then arises could such a cellular state be present in sub-populations prior to gemcitabine exposure and then be selected to produce the same resistance effect but with greater stability given the genetic pre-determination. Three of the selected clone lines (Suit-2R, Suit-2R3, Panc-R) did have high levels of ribonucleotide reductase, at initial review this could suggest convergent evolution with a single mechanism of resistance. However, two clonal cell lines had low levels of ribonucleotide reductase, making it obvious that the clonal populations are not just reliant on a single mechanism. Further analysis elucidated yet more differences and at least four resistance mechanisms can be seen:

A. Significantly increased RRM1 to maintain or increase nucleotide pools in order to overcome replicative stress (Suit-2G+)

B. Prevent S-phase entry through:

1. Encouraging a pause at the G1/S transition by starving the cell of nucleotides due to low ribonucleotide reductase and reduced level of Cyclin D1 (Suit-2R)

2. Either through low levels of Cyclin D1 or cell cycle arrest perhaps via Chk1 whilst ameliorating replicative stress with an elevated level of RRM1 providing nucleotides for completion of S-phase once entered (Suit-2R2)

C. Drive through S-phase accepting replicative stress and arrest or pause at the G2/M checkpoint in order to mitigate failing mitosis. With or without high RRM1 to overcome replicative stress (Suit-2R3, Suit-2R4)

D. Similar to A with developing a measured and controlled transition through the phases with high levels of ribonucleotide reductase to compensate for replicative stress, but in this case based on genetic or epigenetic programming (Panc-R)

These resistance mechanisms in the clonal populations is not surprising given the significant heterogeneity in many types of malignancies, and so it is to be expected that there will be no singular process to ensure survival, even within a single immortalised cell line. A cell's ability to maintain DNA replication and mitosis is fundamental to survival, whether it is through high volume division or measured slow growth. These strategies lead to dramatically different degrees of resistance as well as growth rates. The ability to regulate cell cycle progression is dependent on a complex interwoven network of many different proteins, and a cancer cell already has aberrant functionality to survive under conditions the parent cell would not tolerant. Suit-2 and Panc-1 cell lines are able to survive whether gemcitabine is given as part of culture medium or as a pulsed regimen, and undergo further adaptations once the drug has been removed. There is a versatility that cannot be underestimated because pancreatic cancer cells are able to endure a significant amount of cytotoxicity and still be able to control their cell cycle, as has been shown. The only strategy to cause widespread cell death is to treat with high doses of gemcitabine at a higher frequency which is likely to kill all other cells, ultimately detrimental to a patient.

4.4 Clinical applicability

The chemotherapy treatment of a patient with pancreatic cancer is a staggered approach. Given there is toxicity to all cells and not just the tumour, high doses at frequent intervals are simply unfeasible. The paced approach allows recovery for all cells and potentially the tumour and, if a solid mass is exposed to gemcitabine, there will certainly be a degree of cell death. There are clinical considerations to how drugs are administered and the ability to deliver medication as directly as possible to the tumour itself. Pancreatic cancer has a particular high fibrotic component, potentially restricting access of the drug to the cancer cells. It is unlikely, though not impossible, to cause death to all tumour cells without collateral damage. Repeated exposure to chemotherapy will potentially eliminate more and more cancer cells, and the cells that remain have an opportunity to either adapt to survive the next wave of toxicity. They are selected for because of an innate ability to repair DNA damage and regulate their cell cycle. It is tempting to state that the clonal isolation resistance technique is more realistic to modern chemotherapy regimens, but a solid tumour is not equivalent to a monolayer of cancer cells on a plate. If a patient has a surgical excision of cancerous lesion, then chemotherapy may be acting upon single cells or small clusters of remnant cells in the local

environment or seeded elsewhere. Selection of resistance in a solid tumour, as with the adaptive technique, would allow survival of cancer cells that in themselves are not resistant, but which are protected by surrounding cells, in the case of a tumour both cancer and normal cells. Thus both methods of developing resistant pancreatic cancer cell lines have clinical relevance and neither is an ideal representation.

If a second chemotherapy agent was added to the treatment of pancreatic cancer, then a cell would have to withstand two potentially cytotoxic processes, providing the medications had different mechanisms of action. This double insult would make it significantly harder to survive, which applies equally to normal cells. The considerations above regarding cell exposure and survival would have to be applied to two separate mechanisms of action. The chemotherapy regime would require appropriate dosing and timing to account for complications of the additional chemotherapy agent. This was certainly the outcome of the ESPAC-4 trial which found that an additional 2.5 months of survival was achieved with the addition of capecitabine to gemcitabine chemotherapy. Consequently 608 adverse outcomes were reported in the dual drug cohort compared to 481 in the gemcitabine group (5). There is a trend for multiple chemotherapy regimens in the treatment of many cancers, as demonstrated by the current trials of FOLFIRINOX in pancreatic cancer (27, 32). As mentioned above, a broad-based strategy to chemotherapy is a blunt tool and a more precise tailored approach would be beneficial for the patient and overall survival.

4.5 A personalised approach

From the experiments above, it would be reasonable to select RRM1 and Cyclin D1 as markers of gemcitabine resistance. In an ideal world an increase or decrease in one of these markers could theoretically determine what type of gemcitabine resistance, if any, the cells had acquired. That way unnecessary adverse effects and time wasted could easily be avoided if we had an effective way of dealing with different forms of resistance.

A group from Japan performed similar resistance development experiments in BxPC-3 cells and found high RRM1 expression should be targeted by non-gemcitabine therapies (58). Our own group reviewed tumour tissue of 303 patients from the ESPAC trials and found that intratumoural RRM1 expression is not related to survival (85). And yet there have been retrospective studies of trials and a meta-analysis of large numbers of pancreatic patients who received gemcitabine, reporting that high RRM1 expression leads to poorer survival (46, 86, 87). There has been significantly less work carried out on Cyclin D1. A study of 425 pancreatic ductal cancer patients discovering a variation in

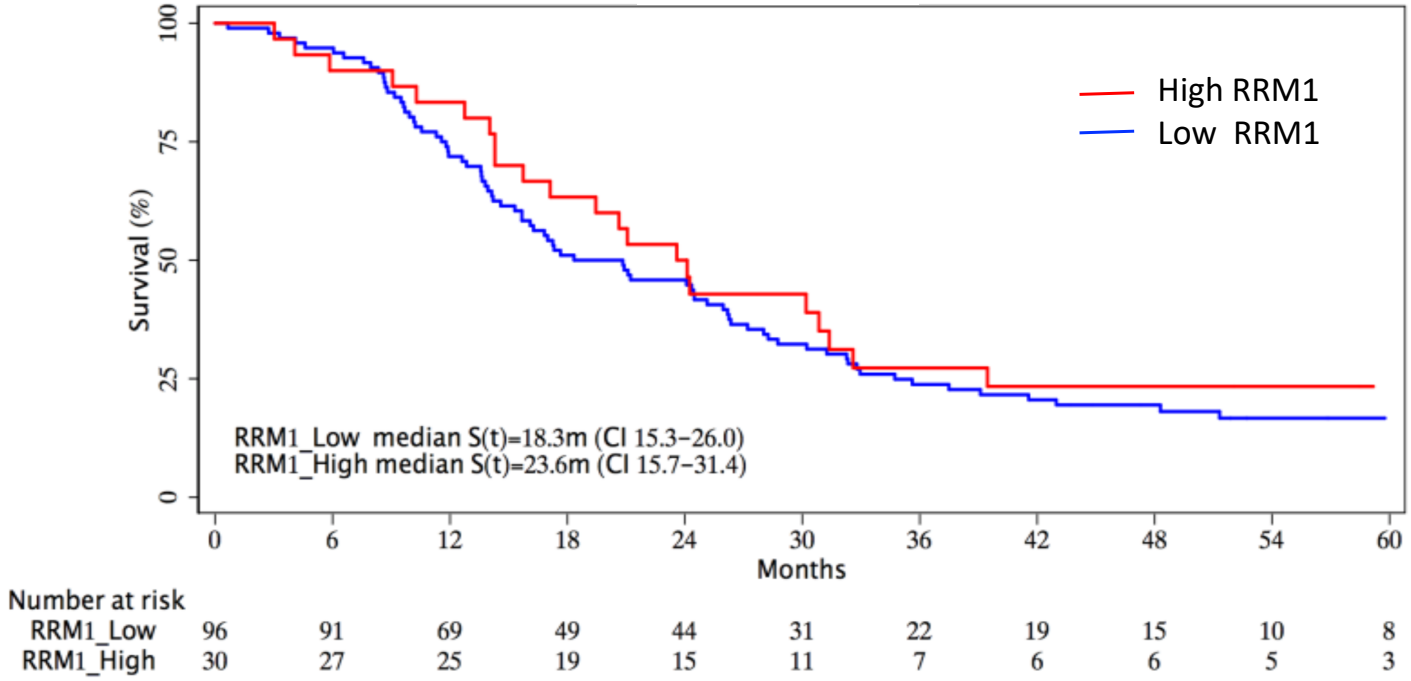
survival in patients who have heterozygous/homozygous forms of the Cyclin D1 G870A polymorphism. The minor allele was found to be associated with poorer survival (72). Which on the basis of this thesis could be explained by Cyclin D1b related resistance.

There are many aspects that need to be considered when interpreting all of this clinical data, including retrospective design, geographical variations, baseline cohort standardisation, experimental quality assurance, and protein thresholds employed. The conclusions derived from cell lines in this thesis have suggested that expression of RRM1 can be used to identify multiple individual forms of resistance, some with high and some with low levels of the protein. Assuming a similar variety exists in patients, studies of populations would result in confusion. High RRM1 indicating resistance in one individual and low RRM1 indicating resistance in another, thus poor survival associated with high or low RRM1 expression. Consequently, RRM1 expression in a population will be deemed to be not associated at all with survival. This possibility would explain the contradictions seen in the literature.

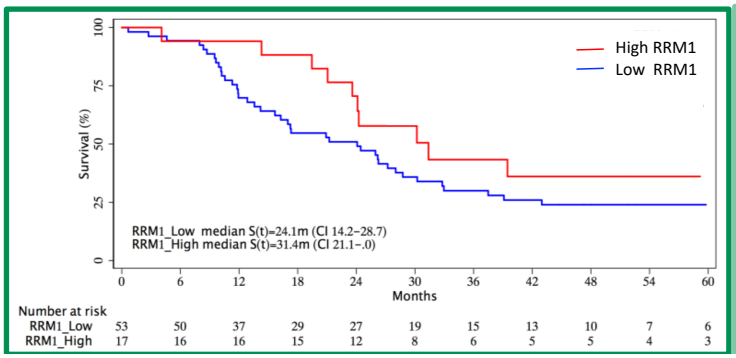
The role of hENT1 has been extensively studied in our unit, leading to publications that have suggested that pancreatic cancer patients with low expression of the transporter should not receive adjuvant gemcitabine chemotherapy. Multiple publications, in the form of retrospective studies and systematic reviews, have stated that increased hENT1 predicts a higher survival in patients with pancreatic cancer (42, 46, 88, 89). Note there have been no validated prospective studies reviewing hENT1 expression. It is logical to assume that gemcitabine delivery may impact the resistance process, such that cell lines will have low dsDNA damage, especially when there is low RRM1 expression. It would be a reasonable argument that intracellular gemcitabine concentration and supply of nucleotide pools would affect the effectiveness of gemcitabine. Thus, it could be hypothesized that by combining RRM1 and hENT1 expression, we may be able to predict patients that would benefit from this chemotherapy agent. Figure 48 depicts unpublished survival data from ESPAC3 patients that underwent adjuvant chemotherapy with gemcitabine, stratified by intra-tumoural expression of hENT1 and RRM1. This data is included within this thesis with the permission of the pancreas research group at the University of Liverpool.

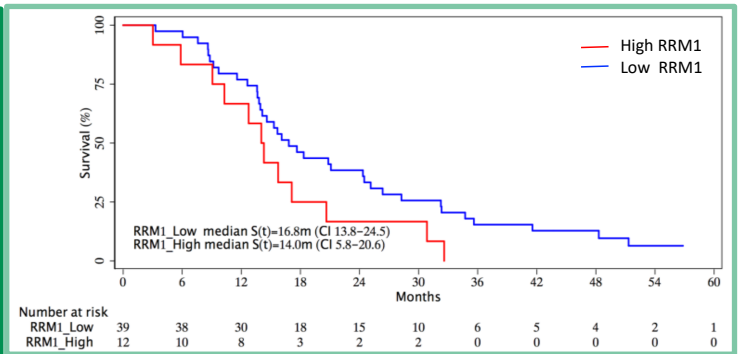
Kulbir Mann: Discussion

a) Overall survival according to RRM1 expression

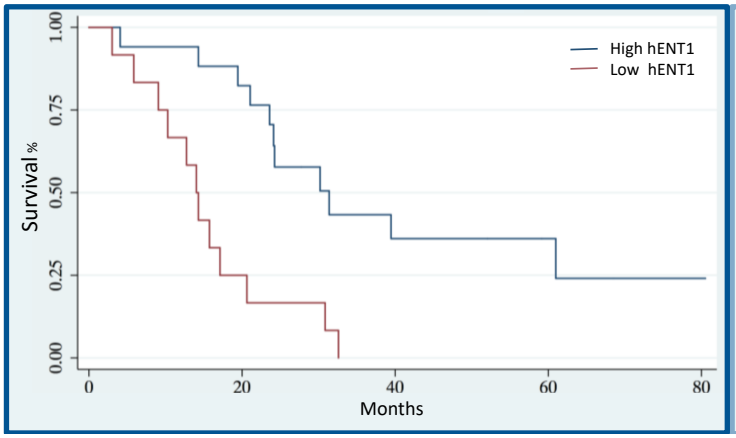


b) Survival with high hENT1, stratified by RRM1 expression c) Survival with low hENT1, stratified by RRM1 expression





d) Survival with high RRM1, stratified to hENT1 expression e) Survival with low RRM1, stratified to hENT1 expression



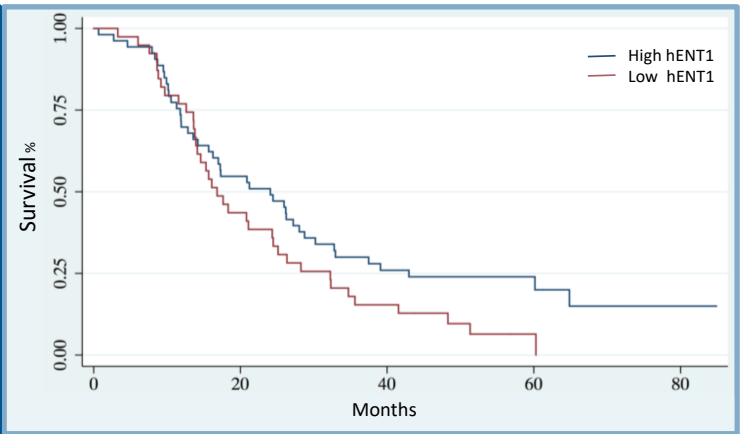


Figure 48 *Kaplan Meier curves to demonstrate overall survival in ESPAC-3 (ver 2) pancreatic cancer patients treated with adjuvant gemcitabine. a) Overall survival correlating to RRM1 level, b) & c) High/Low hENT1 expression stratified by RRM1 expression, d) & e) High/Low RRM1 expression stratified by hENT1 expression. Figure (a) depicts no difference between RRM1 alone but in tumours with high hENT1 where there are higher levels of gemcitabine, there is not only generally increased survival (b,c) but a poorer prognosis if RRM1 is high (c). This suggests tumours are able to undergo DNA replication given that there is less gemcitabine causing toxicity, driving the cell cycle forward. This is also shown in figure d where low hENT1 and high RRM1 causes a stark reduction in overall survival. This effect is not seen with low RRM1 at any level of hENT1 (b,e)*

Even though RRM1 alone shows no change to overall survival when it is correlated with hENT1 expression, there are still prominent changes that deserve exploration. If gemcitabine delivery to a cell is reduced through low hENT1 expression then malignant potential remains, and if the nucleotide pool is maintained through high RRM1 expression then the cell is able to replicate with potential DNA damage. The damage is minimised because the gemcitabine level is low. Patients whose cancer exhibits low RRM1, at whatever level of hENT1, potentially have cancer cells that are unable to enter S-phase, because of starvation of nucleotides. Thus, resistance cells can survive until gemcitabine is degraded. Figure 48e depicts increased survival in patients with high hENT1 in cells with low RRM1, as the high level of intracellular gemcitabine will overwhelm the ribonucleotide reductase, though this effect is not statistically significant.

There is certainly a potential to stratify the administration of gemcitabine monotherapy according to intra-tumoural levels of hENT1 and RRM1. On the basis of this thesis, this could be taken further by reviewing the allele type of the G870A polymorphism (indicating the relative levels of D1a and D1b), and the absolute level of Cyclin D1. This may not only select patients that would benefit most from gemcitabine but also those who would require an additional chemotherapy agent. The only way to pursue this hypothesis would be a blinded randomised control trial with biomarker stratification.

4.6 Future considerations

A couple of studies have specifically observed changes in RRM1 with adjunctive chemotherapy agents in conjunction with gemcitabine. Deferasirox is an oral iron chelator and significantly reduces the expression of RRM1 and RRM2 when given with gemcitabine in mouse models. It was found that increased apoptotic indices were achieved, suggesting increased therapeutic potential of the chemotherapy combination, though an exact synergistic mechanism was not been established (90). The selective MEK1/2 inhibitor, Pimasertib functions by halting the commonly aberrant RAS/RAF pathway from the mutant KRAS gene, found in pancreatic cancer. Its antiproliferative activity stems from halting the phosphorylation process and activation of ERK, which activates downstream targets of cell cycle regulators, of which one interestingly is Cyclin D. The group discovered a reduction in RRM1 in vitro and in vivo and discovered slower tumour growth when Pimasertib was combined with gemcitabine. This synergistic mechanism certainly is in accordance the principles of cell cycle dysregulation, affording an increase in the cytotoxic and cytostatic effects of gemcitabine (91). A Chk1 inhibitor has been used in conjunction with gemcitabine, on the basis that abrogation of the S and G2/M checkpoints would contribute to the cytotoxicity of gemcitabine. They found using in vitro

pancreatic cancer cells that there was reduced expression of RRM1 which sensitised the cells to gemcitabine (76). The use of Chk1 inhibition has been theorised as a target in pancreatic cancer frequently and this thesis has shown some resistant cell lines that express high levels of Chk1 protein (77, 78). This may be a checkpoint marker that could be inhibited for synergistic and cumulative tumour toxicity with gemcitabine.

There are many studies in pancreatic cancer that have discovered multiple cellular adaptations in order to sustain malignant potential. This thesis has demonstrated a multifaceted approach to gemcitabine resistance dependent on the cells ability to repair DNA and regulate its cell cycle. There are many checkpoint markers and cell phase transition recruiters whose expression, in either direction, may increase malignant potential. It is difficult to pinpoint specific proteins that may affect widescale change, especially in cells that are adapting to chemotherapy. A second agent may be the answer to target resistance, as is the current trend of multiple agent regimens. But a balance has to be achieved with patient toleration and degree of tumour specificity. The biomarkers discussed in this thesis will allow further stratification in trials of gemcitabine based therapy combinations.

Bibliography

1. Garden OJ, Parks RW. Hepatobiliary and pancreatic surgery. 6th ed. ed. Edinburgh ; New York: Saunders Elsevier; 2018.

2. Ilic M, Ilic I. Epidemiology of pancreatic cancer. World journal of gastroenterology : WJG. 2016;22(44):9694-705.

3. Ghaneh P, Palmer DH, Cicconi S, Halloran C, Psarelli EE, Rawcliffe CL, et al. ESPAC-5F: Four-arm, prospective, multicenter, international randomized phase II trial of immediate surgery compared with neoadjuvant gemcitabine plus capecitabine (GEMCAP) or FOLFIRINOX or chemoradiotherapy (CRT) in patients with borderline resectable pancreatic cancer. Journal of Clinical Oncology. 2020;38(15_suppl):4505-.

4. Neoptolemos JP, Moore MJ, Cox TF, Valle JW, Palmer DH, McDonald AC, et al. Effect of adjuvant chemotherapy with fluorouracil plus folinic acid or gemcitabine vs observation on survival in patients with resected periampullary adenocarcinoma: the ESPAC-3 periampullary cancer randomized trial. Jama. 2012;308(2):147-56.

5. Neoptolemos JP, Palmer DH, Ghaneh P, Psarelli EE, Valle JW, Halloran CM, et al. Comparison of adjuvant gemcitabine and capecitabine with gemcitabine monotherapy in patients with resected pancreatic cancer (ESPAC-4): a multicentre, open-label, randomised, phase 3 trial. Lancet. 2017;389(10073):1011-24.

6. Neoptolemos JP, Stocken DD, Friess H, Bassi C, Dunn JA, Hickey H, et al. A randomized trial of chemoradiotherapy and chemotherapy after resection of pancreatic cancer. The New England journal of medicine. 2004;350(12):1200-10.

7. Ferlay J, Partensky C, Bray F. More deaths from pancreatic cancer than breast cancer in the EU by 2017. Acta oncologica. 2016;55(9-10):1158-60.

8. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA: a cancer journal for clinicians. 2018;68(6):394-424.

9. Maisonneuve P. Epidemiology and burden of pancreatic cancer. Presse Med. 2019;48(3 Pt 2):e113-e23.

10. Rawla P, Sunkara T, Gaduputi V. Epidemiology of Pancreatic Cancer: Global Trends, Etiology and Risk Factors. World J Oncol. 2019;10(1):10-27.

11. Brierley JGW, C. TNM Classification of Malignant Tumours, 8th Edition: Wiley-Blackwell; 2016.

12. Allemani C, Matsuda T, Di Carlo V, Harewood R, Matz M, Niksic M, et al. Global surveillance of trends in cancer survival 2000-14 (CONCORD-3): analysis of individual records for 37 513 025 patients diagnosed with one of 18 cancers from 322 population-based registries in 71 countries. Lancet. 2018;391(10125):1023-75.

13. Huang Z, Liu F. Diagnostic value of serum carbohydrate antigen 19-9 in pancreatic cancer: a meta-analysis. Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine. 2014;35(8):7459-65.

14. Xing H, Wang J, Wang Y, Tong M, Hu H, Huang C, et al. Diagnostic Value of CA 19-9 and Carcinoembryonic Antigen for Pancreatic Cancer: A Meta-Analysis. Gastroenterology research and practice. 2018;2018:8704751.

15. Hasan S, Jacob R, Manne U, Paluri R. Advances in pancreatic cancer biomarkers. Oncol Rev. 2019;13(1):410.

16. Ballehaninna UK, Chamberlain RS. The clinical utility of serum CA 19-9 in the diagnosis, prognosis and management of pancreatic adenocarcinoma: An evidence based appraisal. Journal of gastrointestinal oncology. 2012;3(2):105-19.

17. Kondo N, Murakami Y, Uemura K, Nakagawa N, Takahashi S, Ohge H, et al. Comparison of the prognostic impact of pre- and post-operative CA19-9, SPan-1, and DUPAN-II levels in patients with pancreatic carcinoma. Pancreatology : official journal of the International Association of Pancreatology. 2017;17(1):95-102.

18. Smith RA, Bosonnet L, Ghaneh P, Raraty M, Sutton R, Campbell F, et al. Preoperative CA19-9 levels and lymph node ratio are independent predictors of survival in patients with resected pancreatic ductal adenocarcinoma. Digestive surgery. 2008;25(3):226-32.

19. Chu LC, Goggins MG, Fishman EK. Diagnosis and Detection of Pancreatic Cancer. Cancer J. 2017;23(6):333-42.

20. Lee ES, Lee JM. Imaging diagnosis of pancreatic cancer: a state-of-the-art review. World journal of gastroenterology : WJG. 2014;20(24):7864-77.

21. O'Reilly D, Fou L, Hasler E, Hawkins J, O'Connell S, Pelone F, et al. Diagnosis and management of pancreatic cancer in adults: A summary of guidelines from the UK National Institute for Health and Care Excellence. Pancreatology : official journal of the International Association of Pancreatology. 2018;18(8):962-70.

22. Zamboni GA, Kruskal JB, Vollmer CM, Baptista J, Callery MP, Raptopoulos VD. Pancreatic adenocarcinoma: value of multidetector CT angiography in preoperative evaluation. Radiology. 2007;245(3):770-8.

23. Ghaneh P, Hanson R, Titman A, Lancaster G, Plumpton C, Lloyd-Williams H, et al. PET-PANC: multicentre prospective diagnostic accuracy and health economic analysis study of the impact of combined modality 18fluorine-2-fluoro-2-deoxy-d-glucose positron emission tomography with computed tomography scanning in the diagnosis and management of pancreatic cancer. Health Technol Assess. 2018;22(7):1-114.

24. Puli SR, Bechtold ML, Buxbaum JL, Eloubeidi MA. How good is endoscopic ultrasound-guided fine-needle aspiration in diagnosing the correct etiology for a solid pancreatic mass?: A meta-analysis and systematic review. Pancreas. 2013;42(1):20-6.

25. Cancer Research UK. Pancreatic cancer statistics 2019 [Available from: https://www.cancerresearchuk.org/health-professional/cancer-statistics/statistics-by-cancer-type/pancreatic-cancer.

26. Isaji S, Mizuno S, Windsor JA, Bassi C, Fernandez-Del Castillo C, Hackert T, et al. International consensus on definition and criteria of borderline resectable pancreatic ductal adenocarcinoma 2017. Pancreatology : official journal of the International Association of Pancreatology. 2018;18(1):2-11.

27. Conroy T, Hammel P, Hebbar M, Ben Abdelghani M, Wei AC, Raoul JL, et al. FOLFIRINOX or Gemcitabine as Adjuvant Therapy for Pancreatic Cancer. The New England journal of medicine. 2018;379(25):2395-406.

28. Andriulli A, Festa V, Botteri E, Valvano MR, Koch M, Bassi C, et al. Neoadjuvant/preoperative gemcitabine for patients with localized pancreatic cancer: a meta-analysis of prospective studies. Annals of surgical oncology. 2012;19(5):1644-62.

29. Tajima H, Ohta T, Okazaki M, Yamaguchi T, Ohbatake Y, Okamoto K, et al. Neoadjuvant chemotherapy with gemcitabine-based regimens improves the prognosis of node positive resectable pancreatic head cancer. Mol Clin Oncol. 2019;11(2):157-66.

30. Xie L, Xia L, Klaiber U, Sachsenmaier M, Hinz U, Bergmann F, et al. Effects of neoadjuvant FOLFIRINOX and gemcitabine-based chemotherapy on cancer cell survival and death in patients with pancreatic ductal adenocarcinoma. Oncotarget. 2019;10(68):7276-87.

31. Schorn S, Demir IE, Reyes CM, Saricaoglu C, Samm N, Schirren R, et al. The impact of neoadjuvant therapy on the histopathological features of pancreatic ductal adenocarcinoma - A systematic review and meta-analysis. Cancer treatment reviews. 2017;55:96-106.

32. Suker M, Beumer BR, Sadot E, Marthey L, Faris JE, Mellon EA, et al. FOLFIRINOX for locally advanced pancreatic cancer: a systematic review and patient-level meta-analysis. The Lancet Oncology. 2016;17(6):801-10.

33. Versteijne E, Vogel JA, Besselink MG, Busch ORC, Wilmink JW, Daams JG, et al. Meta-analysis comparing upfront surgery with neoadjuvant treatment in patients with resectable or borderline resectable pancreatic cancer. The British journal of surgery. 2018;105(8):946-58.
34. Lambert A, Gavoille C, Conroy T. Current status on the place of FOLFIRINOX in metastatic pancreatic cancer and future directions. Therapeutic advances in gastroenterology. 2017;10(8):631-45.
35. Goldstein D, El-Maraghi RH, Hammel P, Heinemann V, Kunzmann V, Sastre J, et al. nab-Paclitaxel plus gemcitabine for metastatic pancreatic cancer: long-term survival from a phase III trial. Journal of the National Cancer Institute. 2015;107(2).
36. Binenbaum Y, Na'ara S, Gil Z. Gemcitabine resistance in pancreatic ductal adenocarcinoma. Drug Resist Updat. 2015;23:55-68.
37. Wang L, Bernards R. Taking advantage of drug resistance, a new approach in the war on cancer. Front Med. 2018;12(4):490-5.
38. Paproski RJ, Young JD, Cass CE. Predicting gemcitabine transport and toxicity in human pancreatic cancer cell lines with the positron emission tomography tracer 3'-deoxy-3'-fluorothymidine. Biochemical pharmacology. 2010;79(4):587-95.
39. Zhang GB, Chen J, Wang LR, Li J, Li MW, Xu N, et al. RRM1 and ERCC1 expression in peripheral blood versus tumor tissue in gemcitabine/carboplatin-treated advanced non-small cell lung cancer. Cancer chemotherapy and pharmacology. 2012;69(5):1277-87.
40. Elander NO, Aughton K, Ghaneh P, Neoptolemos JP, Palmer DH, Cox TF, et al. Expression of dihydropyrimidine dehydrogenase (DPD) and hENT1 predicts survival in pancreatic cancer. British journal of cancer. 2018;118(7):947-54.
41. Greenhalf W, Ghaneh P, Neoptolemos JP, Palmer DH, Cox TF, Lamb RF, et al. Pancreatic cancer hENT1 expression and survival from gemcitabine in patients from the ESPAC-3 trial. Journal of the National Cancer Institute. 2014;106(1):djt347.
42. Bird NT, Elmasry M, Jones R, Psarelli E, Dodd J, Malik H, et al. Immunohistochemical hENT1 expression as a prognostic biomarker in patients with resected pancreatic ductal adenocarcinoma undergoing adjuvant gemcitabine-based chemotherapy. The British journal of surgery. 2017;104(4):328-36.
43. Nakano T, Saiki Y, Kudo C, Hirayama A, Mizuguchi Y, Fujiwara S, et al. Acquisition of chemoresistance to gemcitabine is induced by a loss-of-function missense mutation of DCK. Biochemical and biophysical research communications. 2015;464(4):1084-9.

44. Saiki Y, Yoshino Y, Fujimura H, Manabe T, Kudo Y, Shimada M, et al. DCK is frequently inactivated in acquired gemcitabine-resistant human cancer cells. Biochemical and biophysical research communications. 2012;421(1):98-104.

45. Hu Q, Qin Y, Xiang J, Liu W, Xu W, Sun Q, et al. dCK negatively regulates the NRF2/ARE axis and ROS production in pancreatic cancer. Cell Prolif. 2018;51(4):e12456.

46. Sierzega M, Pach R, Kulig P, Legutko J, Kulig J. Prognostic Implications of Expression Profiling for Gemcitabine-Related Genes (hENT1, dCK, RRM1, RRM2) in Patients With Resectable Pancreatic Adenocarcinoma Receiving Adjuvant Chemotherapy. Pancreas. 2017;46(5):684-9.

47. Elebro J, Ben Dror L, Heby M, Nodin B, Jirstrom K, Eberhard J. Prognostic effect of hENT1, dCK and HuR expression by morphological type in periampullary adenocarcinoma, including pancreatic cancer. Acta oncologica. 2016;55(3):286-96.

48. Gandhi V, Legha J, Chen F, Hertel LW, Plunkett W. Excision of 2',2'-difluorodeoxycytidine (gemcitabine) monophosphate residues from DNA. Cancer research. 1996;56(19):4453-9.

49. Ceppi P, Volante M, Novello S, Rapa I, Danenberg KD, Danenberg PV, et al. ERCC1 and RRM1 gene expressions but not EGFR are predictive of shorter survival in advanced non-small-cell lung cancer treated with cisplatin and gemcitabine. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2006;17(12):1818-25.

50. Heo SJ, Jung I, Lee CK, Kim JH, Lim SM, Moon YW, et al. A randomized phase II trial of ERCC1 and RRM1 mRNA expression-based chemotherapy versus docetaxel/carboplatin in advanced non-small cell lung cancer. Cancer chemotherapy and pharmacology. 2016;77(3):539-48.

51. Pourquier P, Gioffre C, Kohlhagen G, Urasaki Y, Goldwasser F, Hertel LW, et al. Gemcitabine (2',2'-difluoro-2'-deoxycytidine), an antimetabolite that poisons topoisomerase I. Clinical cancer research : an official journal of the American Association for Cancer Research. 2002;8(8):2499-504.

52. Ju HQ, Gocho T, Aguilar M, Wu M, Zhuang ZN, Fu J, et al. Mechanisms of Overcoming Intrinsic Resistance to Gemcitabine in Pancreatic Ductal Adenocarcinoma through the Redox Modulation. Molecular cancer therapeutics. 2015;14(3):788-98.

53. Pramanik KC, Makena MR, Bhowmick K, Pandey MK. Advancement of NF-kappaB Signaling Pathway: A Novel Target in Pancreatic Cancer. International journal of molecular sciences. 2018;19(12).

54. Heinemann V, Xu YZ, Chubb S, Sen A, Hertel LW, Grindey GB, et al. Inhibition of ribonucleotide reduction in CCRF-CEM cells by 2',2'-difluorodeoxycytidine. Molecular pharmacology. 1990;38(4):567-72.

55. Minami K, Shinsato Y, Yamamoto M, Takahashi H, Zhang S, Nishizawa Y, et al. Ribonucleotide reductase is an effective target to overcome gemcitabine resistance in gemcitabine-resistant pancreatic cancer cells with dual resistant factors. J Pharmacol Sci. 2015;127(3):319-25.
56. Nakahira S, Nakamori S, Tsujie M, Takahashi Y, Okami J, Yoshioka S, et al. Involvement of ribonucleotide reductase M1 subunit overexpression in gemcitabine resistance of human pancreatic cancer. International journal of cancer Journal international du cancer. 2007;120(6):1355-63.
57. Nakano Y, Tanno S, Koizumi K, Nishikawa T, Nakamura K, Minoguchi M, et al. Gemcitabine chemoresistance and molecular markers associated with gemcitabine transport and metabolism in human pancreatic cancer cells. British journal of cancer. 2007;96(3):457-63.
58. Yoneyama H, Takizawa-Hashimoto A, Takeuchi O, Watanabe Y, Atsuda K, Asanuma F, et al. Acquired resistance to gemcitabine and cross-resistance in human pancreatic cancer clones. Anti-cancer drugs. 2015;26(1):90-100.
59. Wang C, Zhang W, Fu M, Yang A, Huang H, Xie J. Establishment of human pancreatic cancer gemcitabineresistant cell line with ribonucleotide reductase overexpression. Oncology reports. 2015;33(1):383-90.
60. Chen J, Li S, Xiao Y, Zou X, Zhang X, Zhu M, et al. p53R2 as a novel prognostic biomarker in nasopharyngeal carcinoma. BMC cancer. 2017;17(1):846.
61. Sato J, Kimura T, Saito T, Anazawa T, Kenjo A, Sato Y, et al. Gene expression analysis for predicting gemcitabine resistance in human cholangiocarcinoma. Journal of hepato-biliary-pancreatic sciences. 2011;18(5):700-11.
62. Yousefi B, Rahmati M, Ahmadi Y. The roles of p53R2 in cancer progression based on the new function of mutant p53 and cytoplasmic p21. Life sciences. 2014;99(1-2):14-7.
63. Cho EC, Yen Y. Novel regulators and molecular mechanisms of p53R2 and its disease relevance. Biochimie. 2016;123:81-4.
64. Abbas T, Dutta A. p21 in cancer: intricate networks and multiple activities. Nature reviews Cancer. 2009;9(6):400-14.
65. Wang J, Zhu Y, Chen J, Yang Y, Zhu L, Zhao J, et al. Identification of a novel PAK1 inhibitor to treat pancreatic cancer. Acta Pharm Sin B. 2020;10(4):603-14.
66. Moon SU, Kim JW, Sung JH, Kang MH, Kim SH, Chang H, et al. p21-Activated Kinase 4 (PAK4) as a Predictive Marker of Gemcitabine Sensitivity in Pancreatic Cancer Cell Lines. Cancer research and treatment : official journal of Korean Cancer Association. 2015;47(3):501-8.

67. Jagadeeshan S, Subramanian A, Tentu S, Beesetti S, Singhal M, Raghavan S, et al. P21-activated kinase 1 (Pak1) signaling influences therapeutic outcome in pancreatic cancer. Annals of oncology : official journal of the European Society for Medical Oncology / ESMO. 2016;27(8):1546-56.
68. Yeo D, He H, Baldwin GS, Nikfarjam M. The role of p21-activated kinases in pancreatic cancer. Pancreas. 2015;44(3):363-9.
69. Weinberg RA. The retinoblastoma protein and cell cycle control. Cell. 1995;81(3):323-30.
70. Yan L, Wang Y, Wang ZZ, Rong YT, Chen LL, Li Q, et al. Cell motility and spreading promoted by CEACAM6 through cyclin D1/CDK4 in human pancreatic carcinoma. Oncology reports. 2016;35(1):418-26.
71. Zhang Y, Su Y, Zhao Y, Lv G, Luo Y. MicroRNA720 inhibits pancreatic cancer cell proliferation and invasion by directly targeting cyclin D1. Molecular medicine reports. 2017;16(6):9256-62.
72. Bachmann K, Neumann A, Hinsch A, Nentwich MF, El Gammal AT, Vashist Y, et al. Cyclin D1 is a strong prognostic factor for survival in pancreatic cancer: analysis of CD G870A polymorphism, FISH and immunohistochemistry. Journal of surgical oncology. 2015;111(3):316-23.
73. Akhter N, Alzahrani FA, Dar SA, Wahid M, Sattar RSA, Hussain S, et al. AA genotype of cyclin D1 G870A polymorphism increases breast cancer risk: Findings of a case-control study and meta-analysis. Journal of cellular biochemistry. 2019;120(10):16452-66.
74. Xie M, Zhao F, Zou X, Jin S, Xiong S. The association between CCND1 G870A polymorphism and colorectal cancer risk: A meta-analysis. Medicine. 2017;96(42):e8269.
75. Awasthi P, Foiani M, Kumar A. ATM and ATR signaling at a glance. Journal of cell science. 2015;128(23):4255-62.
76. Liang M, Zhao T, Ma L, Guo Y. CHK1 inhibition sensitizes pancreatic cancer cells to gemcitabine via promoting CDK-dependent DNA damage and ribonucleotide reductase downregulation. Oncology reports. 2018;39(3):1322-30.
77. Koh SB, Wallez Y, Dunlop CR, Bernaldo de Quiros Fernandez S, Bapiro TE, Richards FM, et al. Mechanistic Distinctions between CHK1 and WEE1 Inhibition Guide the Scheduling of Triple Therapy with Gemcitabine. Cancer research. 2018;78(11):3054-66.
78. Wallez Y, Dunlop CR, Johnson TI, Koh SB, Fornari C, Yates JWT, et al. The ATR Inhibitor AZD6738 Synergizes with Gemcitabine In Vitro and In Vivo to Induce Pancreatic Ductal Adenocarcinoma Regression. Molecular cancer therapeutics. 2018;17(8):1670-82.

79. Bastin-Coyette L, Cardoen S, Smal C, de Viron E, Arts A, Amsailale R, et al. Nucleoside analogs induce proteasomal down-regulation of p21 in chronic lymphocytic leukemia cell lines. Biochemical pharmacology. 2011;81(5):586-93.
80. Parsels LA, Tanska DM, Parsels JD, Zabludoff SD, Cuneo KC, Lawrence TS, et al. Dissociation of gemcitabine chemosensitization by CHK1 inhibition from cell cycle checkpoint abrogation and aberrant mitotic entry. Cell cycle. 2016;15(5):730-9.
81. Salazar J, Moya P, Altes A, Diaz-Torne C, Casademont J, Cerda-Gabaroi D, et al. Polymorphisms in genes involved in the mechanism of action of methotrexate: are they associated with outcome in rheumatoid arthritis patients? Pharmacogenomics. 2014;15(8):1079-90.
82. Uehara M, Domoto T, Takenaka S, Bolidong D, Takeuchi O, Miyashita T, et al. Glycogen synthase kinase-3beta participates in acquired resistance to gemcitabine in pancreatic cancer. Cancer science. 2020;111(12):4405-16.
83. Xue L, Zhou B, Liu X, Heung Y, Chau J, Chu E, et al. Ribonucleotide reductase small subunit p53R2 facilitates p21 induction of G1 arrest under UV irradiation. Cancer research. 2007;67(1):16-21.
84. Zhou J, Chen Z, Malysa A, Li X, Oliveira P, Zhang Y, et al. A kinome screen identifies checkpoint kinase 1 (CHK1) as a sensitizer for RRM1-dependent gemcitabine efficacy. PloS one. 2013;8(3):e58091.
85. Elander NO, Aughton K, Ghaneh P, Neoptolemos JP, Palmer DH, Cox TF, et al. Intratumoural expression of deoxycytidylate deaminase or ribonuceotide reductase subunit M1 expression are not related to survival in patients with resected pancreatic cancer given adjuvant chemotherapy. British journal of cancer. 2018;118(8):1084-8.
86. Aoyama T, Miyagi Y, Murakawa M, Yamaoku K, Atsumi Y, Shiozawa M, et al. Clinical implications of ribonucleotide reductase subunit M1 in patients with pancreatic cancer who undergo curative resection followed by adjuvant chemotherapy with gemcitabine. Oncology letters. 2017;13(5):3423-30.
87. Han QL, Zhou YH, Lyu Y, Yan H, Dai GH. Effect of ribonucleotide reductase M1 expression on overall survival in patients with pancreatic cancer receiving gemcitabine chemotherapy: A literature-based meta-analysis. Journal of clinical pharmacy and therapeutics. 2018;43(2):163-9.
88. Nordh S, Ansari D, Andersson R. hENT1 expression is predictive of gemcitabine outcome in pancreatic cancer: a systematic review. World journal of gastroenterology : WJG. 2014;20(26):8482-90.

89. Yabushita Y, Mori R, Taniguchi K, Matsuyama R, Kumamoto T, Sakamaki K, et al. Combined Analyses of hENT1, TS, and DPD Predict Outcomes of Borderline-resectable Pancreatic Cancer. Anticancer research. 2017;37(5):2465-76.

90. Shinoda S, Kaino S, Amano S, Harima H, Matsumoto T, Fujisawa K, et al. Deferasirox, an oral iron chelator, with gemcitabine synergistically inhibits pancreatic cancer cell growth in vitro and in vivo. Oncotarget. 2018;9(47):28434-44.

91. Vena F, Li Causi E, Rodriguez-Justo M, Goodstal S, Hagemann T, Hartley JA, et al. The MEK1/2 Inhibitor Pimasertib Enhances Gemcitabine Efficacy in Pancreatic Cancer Models by Altering Ribonucleotide Reductase Subunit-1 (RRM1). Clinical cancer research : an official journal of the American Association for Cancer Research. 2015;21(24):5563-77.